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Refractometry of Living Cells

Part II. The Immersion Medium

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(From the Department of Human Anatomy, Oxford)

With one plate (fig. 3)

SUMMARY

This paper continues the discussion of an immersion method of refractometry of living cells, the basic principles of which were given in Part I (Barer and Joseph, 1954). A suitable immersion medium should be non-toxic, must not penetrate the cell, and must be in osmotic equilibrium with it so that no change in volume (and hence in concentration) occurs. These requirements are best met by solutions of substances of high molecular weight. The effects observed when cells become freely permeable to such substances are described. An account of tests with various immersion media is given. The main substances tried have been peptone, proteose, protein hydrolysate, dextran, polyvinyl alcohol, polyvinylpyrrolidone, acacia gum, egg albumin, bovine gamma globulins, carboxyhaemoglobin, and bovine plasma albumin. Of these, bovine plasma albumin has proved to be most generally useful though acacia gum may be a good inexpensive substitute for some cells, particularly fungi.

The osmotic properties of the immersion medium are most important and must be carefully controlled if true determinations of solid concentration are to be made. Some of the many difficulties in defining an isotonic physiological medium are reviewed. It is suggested that the best practical definition is that such a medium should be innocuous and should not change the cell volume. A method of adjusting the salt content of the medium, based on this definition, is described, and the possibility that some cells may exhibit a degree of osmotic regulation is discussed. Finally, evidence is presented to demonstrate the harmlessness of the isotonic protein medium to many types of cells. Photomicrographs of cell division over a period of 48 hours in a protein medium are shown.

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BASIC REQUIREMENTS

AS already indicated the present method depends on matching the refractive index of the cytoplasm of a living cell with that of the immersion medium. The properties of the latter are very important and it is possible to lay down certain conditions which should be fulfilled by the ideal immersion fluid.

(1) Since the purpose of the investigation is to carry out measurements on normal living cells, the immersion medium must be non-toxic and must not affect the structure or function of these cells.

(2) The medium must not penetrate the cell. Should penetration occur even to a limited extent, the refractive index of the cell interior would be changed and an accurate measurement against the external medium would become impossible. The effects obtained when cells become freely permeable to the medium are further discussed below.

(3) The medium must be in osmotic equilibrium with the cell so that the latter remains unaltered in volume. Any swelling or shrinkage would inevitably change the refractive index.

(4) The refractive index of the immersion medium must be variable in small steps or preferably continuously and the range of refractive index covered must include that of the cells to be measured.

Certain conclusions can be drawn from these requirements. In the first place most of the oils and inorganic solvents which are commonly used for the refractometry of crystals and other inert objects are unsuitable for living cells. Even in those few cases where the oil is non-toxic or does not damage the cell (e.g. liquid paraffin) trouble may be caused by a thin film of water which remains in contact with it. The desirability of being able to change the refractive index continuously suggests the use of a solution of variable concentration. The only suitable solvent appears to be water; we have been unable to think of any water-soluble substance of low molecular weight which could be used. Inorganic salts (particularly iodides, which have a high refractive index in solution), urea, sugars, and glycerol were all ruled out because of either toxicity, penetration, or lack of osmotic balance. The most obvious type of substance to use is one of high molecular weight. Such substances exert

very low osmotic pressures so that they can be dissolved in a suitable saline solution in order to make the final solution isotonic with the cells studied. Another advantage of materials of high molecular weight is that their large size and low diffusibility hinder penetration into the cell interior. On the other hand, a rather concentrated solution may be needed to match the refractive index of certain cell structures. This requirement rules out many substances which are insufficiently soluble; the great viscosity of concentrated solutions of some substances of high molecular weight also makes them unsuitable for mechanical reasons. From the practical point of view, therefore, we have attempted to find inert non-toxic substances of molecular weight exceeding 10,000, freely soluble in water, and whose solutions do not exhibit excessive viscosity. Other practical considerations were that the chosen substance should, if possible, be readily available commercially and reasonably inexpensive.

THE PERMEABILITY OF CELLS TO THE IMMERSION MEDIUM

During the early stages of this work some experiments were carried out by Barer and Ross in an attempt to measure the refractive indices of fixed cells. For this purpose hanging drop preparations of spermatocytes of various species were subjected for a short time (generally 1-5 minutes) to the action of osmium tetroxide vapour or formaldehyde vapour. On immersing such cells in a suitable protein medium, it was found to be quite impossible to bring about reversal of contrast, no matter how high the concentration of protein used. Further investigations of a similar nature were then undertaken by the authors, using a variety of cells treated with different physical and chemical agents. As a result of this work it became clear that when cells are treated with certain agents they become freely permeable to bovine plasma albumin so that no matter what the concentration of the latter, the refractive index of the external medium can never exceed that of the cell. What presumably happens in these circumstances is that the aqueous medium within the cell is replaced by a protein solution, so that the total concentration of solids is always slightly higher than that in the external medium. This theory explains the observed fact that when viewed by positive phase-contrast such cells remain dark when immersed in protein, but become progressively paler (though never disappear) as the concentration of protein is increased.

In the case of agents such as precipitant fixatives most of the cell proteins are presumably deposited as a network of rather dense, more or less dehydrated strands, which would be expected to have a refractive index of about 1.54, corresponding to that of many dry protein films and fibres. It is not, of course, possible to immerse cells in a protein medium of such a high refractive index, but the refractive index of fixed and dehydrated cells can be measured very easily by phase-contrast microscopy with non-aqueous immersion media. The use of such media for varying the contrast of unstained tissue sections is a familiar method in phase-contrast microscopy (see Bennett and others,

1951), and Crossmon (1949) employed it to measure the refractive index of fixed sections, obtaining values in the region of 1.536. Unfortunately, a recent paper by Davies and others (1954) gives the impression that the refractive index of a fixed cell cannot be determined by phase-contrast and Crossmon's work is not mentioned; a very much more laborious and less accurate method using interference microscopy is described.

It was first thought that the loss of the permeability barrier against proteins was an inevitable accompaniment of cellular death, but further work has indicated that this is probably not the case. The action of many chemicals, particularly the common fixatives, fat solvents such as ether, alcohol, and chloroform, and many acids and alkalis in sufficient concentration, almost invariably abolishes the permeability barrier even though the gross structure of the cell may be comparatively little affected. Many cells are peculiarly susceptible to slight drying. If, for example, a thin film of mammalian blood is deposited on a slide, a wave of drying may often be seen to pass along the film. If a drop of concentrated protein solution is placed over the boundary region between the moist and drying parts of the film, it is found that most of the cells in the moist region can be reversed in contrast, whereas most of those in the drying region are dark and irreversible. This damage to the permeability properties of the cell by drying can therefore occur with extreme rapidity. The effect is not due to heat as all drying was carried out at room temperature and normally took place within a few seconds of forming a film. Nor did it appear to be related to the surface action of the glass slide, because a high proportion of cells taken from the surface of a large blood-drop exposed to the air also showed loss of the permeability barrier. Surface action at an air-cell interface cannot be ruled out. These observations cast considerable doubt on the validity of a method proposed by Mellors, Kupfer, and Hollender (1953) for measuring the mass and thickness of living cells by multiple-beam interferometry. In this method an air-bubble is manoeuvred to surround a cell and the resulting fringe system is photographed. Experiments carried out here by Dr. D. A. T. Dick with a special irrigation chamber (Dick, 1955) have shown that even the momentary passage of an air-bubble across living chick fibroblasts or snail amoebocytes leaves a track of swollen and damaged cells, some of which are already permeable to protein, and the rest soon become so. The effect is drastic and dramatic and has been described as being 'like the passage of a steam-roller'. It seems unlikely, therefore, that cells observed in an air-bubble could be regarded as viable or even normal in shape.

In contrast to these results autolytic changes were not always accompanied by rapid loss of the permeability barrier. Cells such as spermatocytes, various types of white blood corpuscles, and mouse ascites tumour cells, when kept for several days in protein solutions usually underwent considerable swelling, but still appeared to be impermeable to protein. If the original concentration of protein was sufficiently high to make these cells appear with reversed contrast, they became even more brightly reversed as time went on because

the concentration of solids and hence the refractive index fell with increasing swelling. Frequently, if the concentration of the protein was not quite high enough to cause reversal at first, such reversal did occur after a few days when the cells had swollen enough to reduce their refractive indices below that of the protein. A certain number of cells became dark in the course of time and apparently lost their permeability barrier.

A rather different type of behaviour was seen in mammalian red blood corpuscles. Suspensions of these were made up in protein solutions concentrated enough to produce reversal of contrast, and drops were left overnight between sealed slide-coverslip preparations. By the next day a certain proportion of the cells had become dark and the number of these increased progressively with the passage of time. Comparatively little swelling occurred, but eventually many of the cells haemolysed. A small proportion of the cells seemed to be highly resistant to autolytic changes and still appeared with reversed contrast even after 7 days. A more detailed report on experiments of this type will be given elsewhere. For the moment we may state that there appear to be at least two types of reaction to autolytic changes: in one the cell permeability barrier to proteins and possibly other high-molecular weight compounds is relatively unaffected, at least until a very late stage, and in the other the mechanism or structure responsible for the permeability barrier to such substances is destroyed fairly rapidly. The action of many organic solvents in making cells freely permeable to proteins suggests that lipids may be involved and it is not unlikely that some breakdown in the lipoprotein complexes believed to be present in cell surfaces may be the underlying factor.

The loss of the permeability barrier can in some cases be used as a criterion for cell death and it is therefore of interest to know how sensitive this test is as compared with other criteria. The fact that grossly swollen cells undergoing autolysis can still retain a permeability barrier suggests that the test is not necessarily a very sensitive one, because it is hard to imagine that such cells are alive in the ordinary sense, though of course it is always difficult to define the terms life and death rigorously. There is no special reason why the cell surface should not continue to be impermeable to protein long after other cellular activities generally associated with the living state have ceased. Tests were carried out in collaboration with Dr. G. N. C. Crawford in order to compare the sensitivity of the permeability method with that of vital staining. It was generally found that autolysing cells lost their ability to segregate dyes such as neutral red and became diffusely stained long before the permeability barrier to protein was lost. From this point of view, therefore, the permeability criterion for cell death does not appear to be very delicate. It may, however, be a useful method for studying the permeability of cells to large molecules—a subject about which comparatively little seems to be known. In the following section we shall describe experiments carried out with a variety of substances which were investigated in an attempt to find a suitable alternative to bovine plasma albumin. It will be seen that in many cases cells seemed to be freely permeable to these substances, particularly in high concentrations. In some

cases the presence of diffusible compounds of lower molecular weight may have been responsible for a toxic action accompanied by destruction of the permeability barrier. On the other hand, some of these substances still appeared to penetrate the cells even after they had been subjected to prolonged dialysis, so that the presence of impurities of low molecular weight became unlikely. The mechanism of penetration of such compounds is quite unknown; it is being investigated. It was also found that there were striking differences between the behaviour of the same substances with respect to different types of cells. Gum acacia, for example, did not appear to penetrate the spermatocytes and fibroblasts of several species, whereas it did penetrate the majority of bacteria tested. If a range of different non-penetrating compounds of varying molecular weight were available, it might be possible to assess varying degrees of damage to the permeability barrier. Many of the substances discussed below were investigated from this point of view, but our search has not been completely successful. As regards convenience and general applicability no substitute has been found for bovine plasma albumin. The list of compounds tested is not exhaustive, and no attempt has been made to describe properties of unsuitable substances in detail. Brief mention has been made of some of these, however, in order to assist others who might wish to investigate the properties of possible protein substitutes.

IMMERSION MEDIA OTHER THAN BOVINE PLASMA ALBUMIN

The concentrations of most of the substances discussed here were measured by refractometry, and for convenience they are regarded as having the same refraction increment as protein, so that concentrations are expressed in terms of the equivalent concentration of protein solution having the same refractive index.

Peptone

Many peptone preparations are available commercially. In general they are made from heart or muscle by acid or enzymic digestion, and differ in their content of insoluble material and in the molecular size-range of their components. Thus some preparations were found to contain a high proportion of substances incapable of passing through a collodion membrane, whereas others were almost completely dialysable.

The materials we have mostly used are Armour's numbers 1 and 2, and Evans's bacteriological peptone. The first two gave dark brown, cloudy solutions which had to be clarified by filtration or centrifuging. Solutions of high refractive index corresponding to that of a 50 per cent. protein solution could be prepared without difficulty and were much less viscous. Evans's bacteriological peptone was completely soluble up to at least 50 per cent. and contained virtually no insoluble residue. It appeared to be composed mainly of dialysable substances. The pH of all these solutions lay between pH 4 and pH 5.

Solutions of crude peptones in distilled water were generally unsuitable for work on most of the cells studied, and were evidently very hypertonic. *Amoeba proteus* was rapidly killed by immersion in a 20 per cent. solution, but survived for about 20 minutes in 12 per cent. Resting pseudopodia were bright (i.e. reversed in contrast when positive phase contrast was used) in 7.8 per cent. and dark in 6.3 per cent., in excellent agreement with values found in protein. Thus in low concentrations such as these, crude peptone is not hypertonic and for a time at least is moderately non-toxic. In higher concentrations, however, there is evidence of both hypertonicity and toxicity. We therefore attempted to eliminate salts and other small osmotically active molecules by dialysis. At the same time diffusible substances capable of penetrating living cells and possibly toxic compounds might be removed. Dialyses were carried out as described in Part III for bovine serum albumin and in some cases the pH was also adjusted by dialysis against suitable buffers to between 6.8 and 7.0. The dialysed peptone was frozen-dried and the resulting powder dissolved in water. This material seemed rather less toxic to Protozoa than the crude peptone. Several different types of amoebae survived for periods of up to 30 minutes in concentrations up to 10 per cent. and a few individual *Podophrya* and *Colpidium* were active for periods up to 3 hours, though many died sooner. In these cases valuable confirmation was obtained of the refractive index figures for the same organisms measured in protein solutions.

Experiments on other types of cells were for the most part unsuccessful. It was not possible to cause reversal of contrast in spermatocytes, and red blood corpuscles became grossly distorted even at concentrations considerably below those required to produce reversal in protein. Rather surprisingly it was found that bacteria could not be reversed, though the capsules of some species did become reversed. Some fungal spores, on the other hand, became reversed in very high concentrations, though fungal mycelia could not be reversed.

The unsuitability of crude peptone solutions is not surprising in view of the large proportion of relatively small osmotically active molecules and ions which they contain. The failure of dialysed peptone to cause reversal is less easily explained; it may be that some of the polypeptides present are either toxic or are capable of penetrating living cells. It would not, of course, be surprising to find that such substances are toxic, because after all several antibiotics of the polypeptide group are known, and many natural bacterial toxins are composed of protein. Only three types of peptones were used in these experiments and it may well be that other types may prove to be more suitable.

Proteose

Since trials with peptones were for the most part unsuccessful, an attempt was made to find a protein breakdown-product of higher molecular weight. Pure proteoses are not available, but the commercial preparation Bacto-Protone (Difco) is said to have a high proteose content (5.36 per cent. primary

proteose nitrogen and 7.60 per cent. secondary proteose nitrogen according to the manufacturers). This material contained much insoluble debris which had to be removed by centrifuging. The pH of the supernatant liquid was approximately 6.5. Preliminary tests on several different types of cells were uniformly unsuccessful and further detailed tests were not carried out.

Protein hydrolysate

Several commercial protein hydrolysate preparations can be obtained. Experiments were carried out with Hepamino (Evans), which is a liver hydrolysate. It contains a good deal of insoluble matter which can be removed by centrifuging, leaving a clear brown liquid of pH 6. *Amoeba radiosa* remained motile for some time in 12 per cent., but were not reversed in contrast. *Vorticella* was killed fairly rapidly in 9 per cent. No reversal of tissue cells could be obtained.

Dextran

This is a polysaccharide used for a number of purposes in haematology. It is available in different grades of molecular size. The samples tested were quite unsuitable because of limited solubility and extreme viscosity. Concentrations approaching 20 per cent. formed almost solid pastes.

Polyvinyl alcohol

This is a polymerized vinyl alcohol, again available in various ranges of molecular size. It can be dissolved in hot water to form rather viscous neutral solutions at concentrations up to 25 per cent. Amoebae became reversed and survived for periods up to 30 minutes. *Paramecium* survived for only a short time in 14 per cent. The high viscosity makes this material unsuitable for general purposes, but further work with less polymerized samples may be profitable.

Polyvinylpyrrolidone

This is a synthetic polymerized vinylpyrrolidone which was first employed by Hecht and Weese (1943) as a substitute for blood-plasma in cases of shock. It has since been extensively investigated and used for this purpose, and is said to be reasonably non-toxic. Under the name of Periston it became the standard blood substitute in the German Army and is available in this country as Plasmosan (May & Baker). The molecular weight varies from 30,000 to over 100,000, depending on the degree of polymerization. Samples were obtained from Messrs. May & Baker, Dagenham, Essex, and Messrs. Milwards Merchandise, Dacre House, Victoria Street, London, S.W. 1, and we wish to thank these firms for their generous co-operation. The material is sold as a dry powder which is readily soluble in water. The pH of different samples is said to vary somewhat, but we have usually found it to be about 5.5.

Amoebae were reversed in 15 per cent., but soon contracted and became immobile. Reversal also occurred in 10 per cent., in which the cells survived

for about 30 minutes. Values for the refractive index obtained in polyvinylpyrrolidone agreed with those obtained in dialysed peptone and protein. It was not possible to cause reversal of contrast in bacteria and fungal spores, and red blood corpuscles became grossly distorted in high concentrations. A curious effect, first pointed out by Mr. K. F. A. Ross, was observed in snail spermatocytes. Both the cytoplasm and nucleoplasm of these cells were reversed in contrast by immersion in 20 per cent. solutions. Within a few minutes, however, the cytoplasm became progressively fainter and then dark, indicating that the permeability barrier had been damaged and that polyvinylpyrrolidone had leaked into the cytoplasm. The nucleoplasm, on the other hand, remained reversed in contrast for at least 30 minutes, suggesting that the nucleus possessed a permeability barrier of its own, and was still capable of keeping out polyvinylpyrrolidone. We have found similar effects in spermatocytes of other species, though the time relationships were different.

Attempts to purify the material by dialysis were usually unsuccessful because very great pressure was developed in the membrane. However, one dialysed sample was prepared by using several thicknesses of protective stocking (see Part III) in order to withstand this pressure. The properties of this sample did not, however, appear to differ greatly from those of undialysed solutions. Nor were better results obtained when the pH of the solution was adjusted to neutrality.

This material is thus disappointing as an immersion medium, and its unsuitability is all the more surprising in view of the fact that it has been so widely used clinically. It is only fair to point out that the concentrations employed in clinical practice, namely, about 3.5 per cent. are very much lower than we have used for immersion refractometry, so that gross cell damage is not necessarily likely to occur. Our results do, however, suggest that polyvinylpyrrolidone is capable of leaking into cells and a number of investigations have been reported in which histological changes have been found in various organs after the injection of massive doses of this substance into animals (Ammon and Müller, 1949). Weese (1951) made the interesting observation that after a first injection only about half the amount injected could be accounted for in the blood and very little was excreted. Examination of various organs revealed the presence of polyvinylpyrrolidone in amounts sufficient to account for the quantity injected. He made the significant suggestion that the substance became bound to plasma globulins and cell-wall globulins.

Thrower and Campbell (1951), on the other hand, found evidence of only a little interaction between polyvinylpyrrolidone and some protein films, though they did not investigate globulins. They did, however, observe that the material was slightly surface-active and lowered surface tension. Any interaction between polyvinylpyrrolidone and the proteins of the cell surface might very well account for the effects already described. The low concentrations used for clinical work may be insufficient to produce permanent damage, but in the presence of high concentrations, such as are necessary for immersion

refractometry, considerable disruption of the cell surface may occur on the submicroscopic scale. It may be that the proteins at the surface of the nuclear membrane differ in character and react much more slowly with polyvinylpyrrolidone. These suggestions are highly speculative, however, and much more detailed knowledge is required concerning the possible interaction of polyvinylpyrrolidone (and other substances of high molecular weight) with proteins.

Acacia gum (gum-arabic)

This substance is of considerable historical importance as it was one of the earliest to be employed as a blood substitute (Bayliss, 1917). It is also of special interest in that it was used for immersion refractometry by Fauré-Fremiet (1929), who carried out measurements on the amoebocytes of *Lumbricus*. He did not, however, make any further use of the method, nor did he control the tonicity of the medium.

Acacia gum is a natural product whose structure has been extensively investigated (see Hirst, 1942). It is an acid polysaccharide with a molecular weight in the region of 200,000 (Oakley, 1935). It is available commercially either in granular form or as a powder; the latter is more convenient for making solutions. When added to water, the gum swells at first and then goes into solution. Concentrations up to 50 per cent. can be reached, though with rather more difficulty than in the case of bovine plasma albumin. The viscosity of concentrated solutions, though high, is not excessive for refractometry. The pH is usually about 4.0, but can easily be adjusted to neutrality by the addition of alkali.

Amoebae survived quite well in concentrations below 15 per cent., but were killed in 3–5 minutes in 30 per cent. *Paramecium* remained active for more than 1 hour in 18 per cent., but became immobile and discharged their trichocysts in 30 per cent., though their cilia continued to beat. Reversal of contrast was obtained in spermatocytes and other types of tissue-cells and unlike what was observed with polyvinylpyrrolidone such reversal appeared to be permanent and the cells remained, so far as could be judged, in good condition for some time. Spermatozoa continued to move actively in concentrations up to about 20 per cent. The movements were slower in higher concentrations, possibly because of the greater viscosity. Excellent results were obtained with fungal spores and mycelia even in concentrations up to 50 per cent., and identical values were found for the refractive indices of such material measured both in acacia and in bovine albumin. Germination of fungal spores was observed in acacia solutions without any added nutrient substances. The mycelia from such spores did not, however, attain a great length and no reproductive stages could be seen. This suggests that acacia is non-toxic to fungi, but is incapable of supplying their nutritional requirements. In contrast to this, acacia appeared to be quite unsuitable for use with bacteria, which never became reversed at any concentration. Mammalian red blood-cells too became grossly distorted.

Further purification was attempted by the process of dialysis followed by freezing-drying. The purified material behaved in a very similar manner to the crude gum, though the viscosity seemed to be somewhat less. Spermatozoa appeared to be rather more active and survived longer, but no better results were obtained with bacteria or red blood-cells.

It is thus evident that gum acacia is a promising material for use as an immersion medium for some types of cells. We have in fact used it extensively for work on fungi, for which it does not appear to be inferior to bovine albumin. It is also suitable for many types of tissue-cells which only require comparatively low concentrations not exceeding 25 per cent., but it causes distortion of blood corpuscles and other cells in higher concentrations. So far as can be judged at present, it is not a complete substitute for protein, but it may be adequate for certain purposes. Its great advantage over bovine albumin is its very low cost. It is in fact by far the cheapest of all the substances capable of being used as immersion media. The failure of acacia gum to cause reversal of contrast in bacteria is interesting and suggests that the bacterial cell wall is very different in constitution from that of fungi and tissue cells.

Egg albumin

A small quantity of fairly pure egg albumin was obtained from Dr. A. G. Ogston. This material was found to behave rather similarly to bovine albumin, though extended trials were not possible. Commercial pure egg albumin is prohibitively expensive and has not been investigated. Crude egg albumin is, however, available commercially at a very low price in the form of dried flakes. It is commonly used in histology for attaching sections to slides. Although it seemed likely that such albumin would be grossly denatured, we nevertheless investigated its properties. The flakes go into solution in water fairly readily and concentrations up to 50 per cent. can be attained. The viscosity is lower than that of bovine albumin, but the pH (5.0) is approximately the same. The solutions have a very disagreeable odour. The crude material appears to be hypertonic and toxic. Spermatozoa were killed rather rapidly and spermatocytes became shrunk and distorted. *Paramecium* was killed almost at once in 32 per cent., but some survived for up to 30 minutes in 20 per cent.

The crude egg albumin was purified by dialysis followed by freezing-drying. The resulting material could not be dissolved in distilled water, as a slow precipitation occurred. Satisfactory clear solutions could, however, be made in sodium chloride solutions exceeding about 0.4 per cent. in concentration. Such solutions were much less yellowish than the solutions of crude material, and all trace of the disagreeable odour disappeared. Since the purified egg albumin has to be dissolved in salt solutions, it is not very suitable for work on fresh water protozoa, but it has given good results with tissue-cells, spermatozoa, fungi, and bacteria. It is also one of the very few media which has given results with red blood-cells which are in any way satisfactory. We have not employed purified crude egg albumin very extensively because

much more work is needed in order to determine how far the properties of different samples are constant. Like crude acacia gum it may prove to be a valuable alternative to bovine albumin, though again not quite so generally useful.

Bovine gamma globulins

A commercial preparation of plasma globulins (fraction II) is available from Armour Laboratories. This material has to be dissolved in dilute salt solution and appears to be highly soluble (up to at least 40 per cent.). It has given good results with tissue-cells and blood-cells. It is slightly more expensive than bovine albumin and its only advantage may be that the pH of solutions is a little on the alkaline side of neutrality. Extensive tests have not been carried out.

Carboxyhaemoglobin

As the preliminary observations which led to the development of this technique were carried out on cells immersed in haemoglobin solutions, it seems natural the latter should be used as an immersion medium. Unfortunately oxyhaemoglobin is not very stable and becomes converted fairly rapidly to methaemoglobin. Carboxyhaemoglobin is, however, more stable though less soluble. A sample of frozen-dried carboxyhaemoglobin (of sheep) was kindly supplied by Dr. A. G. Ogston. This went into solution readily, but when examined under the microscope was found to contain numerous minute particles. It is possible that the material may have been partly denatured. The presence of the particles did not greatly interfere with its use for immersion refractometry. Good results were obtained with tissue cells, amoebae, various Protozoa, and particularly with spermatozoa, which survived extremely well for long periods. An important advantage of haemoglobin over bovine albumin may be that its isoelectric point is close to pH 7; on the other hand, its deep red colour is a disadvantage and may make the determination of refractive index difficult when high concentrations are used. The main reason for not using it more extensively, however, is that no suitable preparation seems to be available commercially. Crude technical haemoglobin is available, but this was found to be quite unsatisfactory as it contained much insoluble matter.

Other substances

Several other materials were investigated, but were found to be unsuitable for various reasons. Dextrin and 'soluble' starch were insufficiently soluble. Preparations of pectin, soluble alginates, and methyl cellulose gave excessively viscous solutions. Technical grade blood albumin contained much insoluble matter. None of these materials were examined in detail.

BOVINE PLASMA ALBUMIN

Almost the first substance used as an immersion medium was a preparation of bovine plasma albumin fraction V, manufactured in the form of a powder

by Armour & Co. (Barer and Ross, 1952). This material has been used more than any other and has been found suitable for a very wide range of cells. Indeed no type of cell has yet been found in which it has been impossible to produce reversal of contrast in at least some regions.

The Armour product is prepared according to the method of Cohn and others (1946), which involves successive precipitation of plasma at low temperatures by ethyl alcohol in the presence of acetic acid and sodium acetate. The fifth precipitate contains almost all the plasma albumin. This fraction is further purified and re-precipitated by alcohol at pH 5.2, in order to remove most of the acetic acid. The final material is an amorphous powder which according to the makers' brochure contains 3-5 per cent. globulins, less than 2 per cent. ash, and less than 6 per cent. moisture. More accurate figures were obtained from the Research Division of Armour & Co., who stated that the ash content is usually less than 1 per cent. and the moisture 2-3 per cent. There are usually less than 50 parts per million of heavy metals present. The ash consists mainly of sodium acetate and sodium chloride. Every attempt is made to keep the extraction procedure constant and only minor variations are to be expected between different batches. We have used many batches of fraction V over the last 3 years and with the exception of two batches which gave anomalous results with red blood-cells (though normal results with other types of cells) we have found its properties to be remarkably constant, at least as far as the present technique is concerned.

A crystalline preparation of bovine plasma albumin is also available from Armour & Co. This is almost free from globulins and contains less ash. It is very much more expensive than fraction V and seems to offer no special advantages for refractometry. We have also used human plasma albumin with excellent results.

Fraction V can be dissolved in water or salt solution to give a clear, faintly yellow solution. The pale yellow colour is due to a component with an absorption band at $405\text{ m}\mu$, possibly a flavine or flavoprotein present in very small amounts. The pH of the solution is approximately 5.2-5.5, which is a little higher than the isoelectric point of plasma albumin (5.0). Full details of the method used for making up solutions for different purposes are given in the section on technical methods in Part III. At this stage, however, it is necessary to discuss in some detail the osmotic properties of the immersion medium with particular reference to fraction V.

OSMOTIC PROPERTIES OF THE IMMERSION MEDIUM

If accurate quantitative measurements of concentration are to be made, it is essential that the cell volume should be identical in the immersion medium and in life. This is a condition which applies to all techniques for the determination of concentration of any constituent of living cells, and is not peculiar to refractometry alone. There is no absolute certainty that the cell volume remains unchanged when cells are removed from the body and examined

under the microscope or otherwise manipulated, and so far as can be seen there is no method available for measuring such changes if they occur. All one can do is to take the volume occupied by the cell in some accepted 'physiological' medium as a standard. This sounds simple, but in fact it raises a host of important questions which have received little consideration in the past. In the first place, what is an accepted 'physiological' medium? Many such media have been suggested, varying considerably in salt composition, pH, and osmotic properties. A valuable summary of twelve basic solutions used for mammalian tissue-culture work has been compiled recently by Stewart and Kirk (1954). It is remarkable how these media differ. The sodium chloride content, for example, ranges from 0.68 per cent. to 1.5 per cent. Stewart and Kirk have calculated the total particle concentrations of these media, i.e. the concentrations of ions and molecules after assessing the degree of dissociation of various constituents. This quantity should be proportional to the osmotic effect of the medium. The figure 0.307 M was obtained for human plasma, but although a few of the media approximated to this, the range extended from 0.294 M to 0.534 M. Even solutions advocated by the same workers showed wide variations in concentration. Thus Lewis's media extend from 0.354 M to 0.534 M. The calcium Ringer of Vogelaar and Erlichman (quoted by Stewart and Kirk) has a concentration of 0.455 M whereas that of the magnesium Ringer of the same authors is only 0.294 M. A study of the classical paper of M. R. and W. H. Lewis (1911) reveals even more dramatically what wide variations in salt content are compatible with cell growth. They cultured chick embryo tissues (liver, intestine, kidney, heart, and spleen) in hanging drop preparations, using only salt solutions of known composition. In the majority of these experiments the concentrations of calcium chloride (0.025 per cent.), potassium chloride (0.042 per cent.), and sodium bicarbonate (0.02 per cent.) were kept constant, while the sodium chloride content was varied over a wide range. At one extreme 'good' growth was obtained with 0.45 per cent. sodium chloride, at the other 'extensive' growth was obtained with 1.585 per cent. sodium chloride and 'slight' growth even occurred in 1.6975 per cent. sodium chloride. As Lewis and Lewis themselves point out, 'it is quite remarkable that cells will grow in such widely different solutions where the osmotic pressure must vary considerably'. It will be seen from these figures that chick embryo cells are capable of active growth over about a threefold range of osmotic pressure. It would be interesting to know whether cells grown in such diverse media are similar in volume and general form, but Lewis and Lewis do not discuss this. We are attempting to repeat these experiments in order to see if the solid content of the cytoplasm of such cells is affected by the osmotic pressure of the culture medium.

A study of various 'physiological' media suggested for invertebrates shows even greater variations than in mammalian media. Extensive compilations of chemical analysis of the bloods of many species have been made by Prosser and others (1950), Heilbrunn (1952), and Buck (1953). Such tables show considerable differences both in the relative proportions of constituents and

in the total osmotic concentration. For example, the depression of the freezing-point of the blood of fresh-water molluscs is generally between 0.1° and 0.2° C., whereas that for some fresh-water crustacea exceeds 1° C. Very large differences occur between various species of insects (Buck, 1953), and the salt solutions suggested do not always approximate in composition to that of the blood. Probably few investigators go to the length of making up special media for each individual species; there is a tendency to use so-called 'amphibian' Ringer or 'insect' Ringer, &c., but there is strictly no such thing as an average medium identical in composition with the blood of a wide range of species. The fact that cells survive and even thrive in such media probably only shows that they are capable of adjusting themselves to quite gross changes in composition and osmotic pressure.

Blood and tissue fluids

It seems pedantic to question whether blood is a truly physiological medium, but it must be remembered that the majority of cells are not surrounded by blood, but by tissue fluid, the composition of which is largely unknown. It is generally assumed that tissue fluid resembles lymph (Drinker and Yoffey, 1941), though clear evidence is lacking because of the difficulty in collecting tissue fluid even in large animals.

Lymph and certain other body fluids are said to be basically plasma 'ultra-filtrates' and are generally assumed to have very nearly the same composition as plasma itself, but with less protein. Even if such fluids were true ultra-filtrates, however, their constitution would inevitably differ to some extent from that of plasma because the presence of non-diffusible protein ions would lead to a Donnan equilibrium resulting in a greater concentration of negative ions (Cl^- and HCO_3^-) and a lower concentration of positive ions (Na^+ , K^+) in the ultra-filtrate than in the plasma itself.

The analysis of lymph is more or less that to be expected of an ultra-filtrate. The protein content of lymph for different parts of the body varies slightly and corresponding differences occur in the ionic composition. The chloride content of the thoracic duct lymph of the dog is given by Drinker and Yoffey (1941) as 396 mg. per 100 c.c. as compared with 369 mg. per 100 c.c. for serum. Much larger differences are found in the cerebrospinal fluid. In man the chloride content is 440 mg. per 100 c.c. as compared with 360 mg. per 100 c.c. in plasma. The sodium content is 324 mg. per 100 c.c. as compared with 316 mg. per 100 c.c. for plasma. The calcium content of cerebrospinal fluid, however, is only half that of plasma (Merritt and Fremont-Smith, 1937). It is generally believed that cerebrospinal fluid is not simply ultra-filtrate but in part at least a secretion. A similar opinion is now held concerning the aqueous humour (Duke-Elder and Goldsmith, 1951). The osmotic pressure of aqueous humour is actually greater than that of the blood. Until more exact figures are available for the composition of tissue fluid, the belief that blood constitutes a true physiological medium for tissue-cells must remain an approximation and it is a matter for conjecture whether

the size and behaviour of tissue-cells will necessarily be the same in blood as in life. However, for the present at any rate, blood probably remains the nearest practical approach to a true physiological medium.

The osmotic behaviour of cells

There is an extensive literature which deals with the volume changes undergone by certain types of cells in solutions of different ionic strengths. Unfortunately, nearly all such work has been carried out on only two types of cell, namely, egg-cells (usually echinoderm) and mammalian red blood-cells. Even in the case of this limited material much essential information is lacking. The mammalian red cell is very atypical both as regards its structure and its osmotic behaviour. It appears to be far more sensitive to osmotic changes in the medium than are most tissue-cells or white blood-cells. Echinoderm eggs may be more typical in these respects, but an examination of the literature shows that almost no experiments have been carried out on their volume changes in media which depart only slightly from isotonicity. As a rule volume changes have only been measured in rather grossly hypotonic or hypertonic media with the object of determining the laws connecting osmotic pressure and volume. For some cells the relationship $P(V-b) = \text{constant}$ has been found to hold. P is the osmotic pressure of the solution. V is the cell volume, and b is a characteristic constant which can be regarded as the volume occupied by the osmotically inactive constituents of the cell. b is generally about 20–30 per cent. of the resting cell volume. By using this equation it is possible to extrapolate for osmotic pressures in the region of isotonicity; because of the factor b it will be seen that a given change in P produces a relatively smaller change in V , so that, for example, halving P would not double the cell volume. It is not at all certain, however, in view of the lack of experimental evidence, that this formula can be used for approximately isotonic media. We shall discuss the possibility below that individual cells may be capable of some degree of osmotic regulation in approximately isotonic media.

Shapiro and Parpart (1937) have measured the diameters of human and rabbit leucocytes in media of different osmotic pressures. The volume in Ringer-Locke solution was taken as unity. The results plotted in figure 1 of their paper are said to show a more or less linear relationship between V and $1/P$. On closer inspection, however, it will be seen that V scarcely changes between $1/P = 1$ and $1/P = 1.5$, in other words, over a range in which the osmotic pressure exceeds about two-thirds of the standard pressure. These results are in fact compatible with the hypothesis that regulation of volume takes place over a fairly narrow range of concentrations, but that such regulation breaks down beyond certain limits. Investigations of this sort are not too difficult in the case of spherical cells, the diameters of which can be measured, but there are obvious difficulties in working with irregular cells. Brues and Masters (1936) estimated the volume of fibroblasts and sarcoma cells in tissue culture by regarding them as symmetrical spindles and measuring the

diameter at different points along the length of the cells. The behaviour of these cells was found to be rather variable when they were transferred from plasma to 0.9 per cent. sodium chloride. As a rule they swelled a little, but occasionally showed slight shrinkage. The mean change in volume in fibroblasts was +13.7 per cent., in sarcoma cells +4.3 per cent. The relationship $P(V-b) = \text{constant}$ was found to hold reasonably well with $b = 22$ per cent. for fibroblasts, and $b = 26$ per cent. for sarcoma cells. The change in volume on transferring to a so-called isotonic salt solution is of some significance, and illustrates another of the difficulties inherent in the task of defining a 'physiological' medium. Shear and Fogg (1934) investigated the behaviour of tumour cells and normal cells from various organs when placed in saline media. They found that swelling occurred in so-called isotonic sodium chloride and 'physiological' salines. Swelling even took place in hypertonic solutions! Variation of pH between 5 and 10 did not appear to affect this swelling very much, though it was a little more marked in alkaline media. The addition of potassium, calcium, magnesium, and other salts also made no difference. The only significant finding was that swelling occurred more slowly in serum. Following up this clue, Shear (1935) found that swelling was retarded by egg albumin, gelatin, casein, serum proteins, and acacia. Some of the proteins used were even capable of causing reversal of swelling with return towards normal volumes.

All these facts point to the great difficulty of virtual impossibility of determining the true volume of the cell in life. One can attempt to measure the cell volume in blood or some other natural fluid or one can measure it in some arbitrarily selected salt solution or 'physiological' saline, always remembering that the volume in the latter may not be the same as in blood or in the body.

What is an isotonic solution?

There is much confusion in the literature and particularly in textbooks as to the definition of the term 'isotonic'. It is quite clear that what is often called an *isotonic* solution is really *isosmotic*, i.e. one having the same osmotic pressure or freezing-point depression as some standard medium such as plasma. Osmotic pressure is the physical property which can be measured without reference to the behaviour of living cells. Tonicity, on the other hand, is a biological concept which can only be referred to the properties of living cells. It would, for example, be ridiculous to call a solution of mercuric chloride or potassium cyanide 'isotonic' simply because it had the same osmotic pressure as plasma. The first essential point in discussing tonicity therefore is that the medium must not be injurious to living cells. Even in such cases, however, the special permeability properties of the cell must be taken into account. Thus, to quote a simple example given by Heilbrunn (1952, p. 127), a 0.53 M solution of sodium chloride and a 0.37 M solution of calcium chloride are both isosmotic with sea-water. Despite this, sea-urchin eggs do not change in volume when immersed in the sodium chloride solution, but shrink in the calcium chloride solution. The volume is unaltered in 0.30 M calcium

chloride. The latter solution is thus isotonic (but not isosmotic) for these particular cells, though not necessarily for others. Considerations of this sort lead to a simple comprehensive definition of isotonicity. *Two solutions are said to be isotonic for a given type of cell if (a) they are compatible with life and (b) the cell volume is the same in each.* This definition is independent of variables such as differences in composition, relative proportions of ions, and pH, and it does not depend on the total osmotic pressure of the solutions. This is the only satisfactory practical definition for work on immersion refractometry in which the really important factor is the constancy of cell volume. Osmotic pressures either calculated theoretically or determined by freezing-point measurements may not be entirely satisfactory and we have usually adopted the empirical method of adjusting the salt content of our media until the mean cell volume is the same as in a standard medium. Results obtained in this way have, as a matter of fact, agreed rather well with experimental freezing-point determinations.

Adjustment of tonicity of immersion media

The method used is essentially that developed by Ross (1953) in his study of the volume changes of cells during fixation. We are indebted to Mr. Ross for most of the results quoted in this paragraph. The basis of the method is to determine frequency-polygons for cell size in different media. Cell diameters were determined by means of an eyepiece micrometer. As a rule measurements were carried out on not less than 50 cells in each medium. The first cells tested were the primary spermatocytes of *Helix aspersa*. Attempts to measure the distribution of cell diameter in snail-blood gave variable results, probably because of contamination with mucus and digestive fluids. Hédon-Fleig's solution was then used and gave virtually identical results with those obtained in a simpler medium containing only 0.7 per cent. sodium chloride and 0.02 per cent. calcium chloride. Histograms were then derived for cells in a range of sodium chloride concentrations varying from 0.3 per cent. to 1.0 per cent. A remarkable finding was that the general shape and modal value of the histogram was virtually unaltered over a surprisingly wide range of salt concentration; in fact, between 0.5 per cent. and 0.8 per cent. Significant swelling occurred in 0.4 per cent. and shrinkage in 0.9 per cent. Nuclear diameters were also measured with very similar results. The next stage was to make up a 20 per cent. protein solution of bovine plasma albumin fraction V in distilled water and to determine frequency distribution curves of the same type of cells in this medium. The cells were found to be considerably swollen with a modal value of 24μ for the diameter as compared with 19μ in Hédon-Fleig or 0.7 per cent. saline. This frequency distribution corresponded quite well to those obtained in 0.2 per cent. and 0.15 per cent. sodium chloride (fig. 1). On this evidence, therefore, it appeared that a 10 per cent. fraction V solution in distilled water would be equivalent in tonicity to a sodium chloride concentration of between 0.075 per cent. and 0.1 per cent. Taking the latter value as a convenient round figure, a 20 per cent. protein solution

would require the addition of 0.5 per cent. of sodium chloride in order to make it isotonic with 0.7 per cent. salt solution. Histograms for spermatocytes in 20 per cent. protein in 0.5 per cent. sodium chloride and also in 20 per cent. protein in 0.6 per cent. sodium chloride are shown in fig. 1.

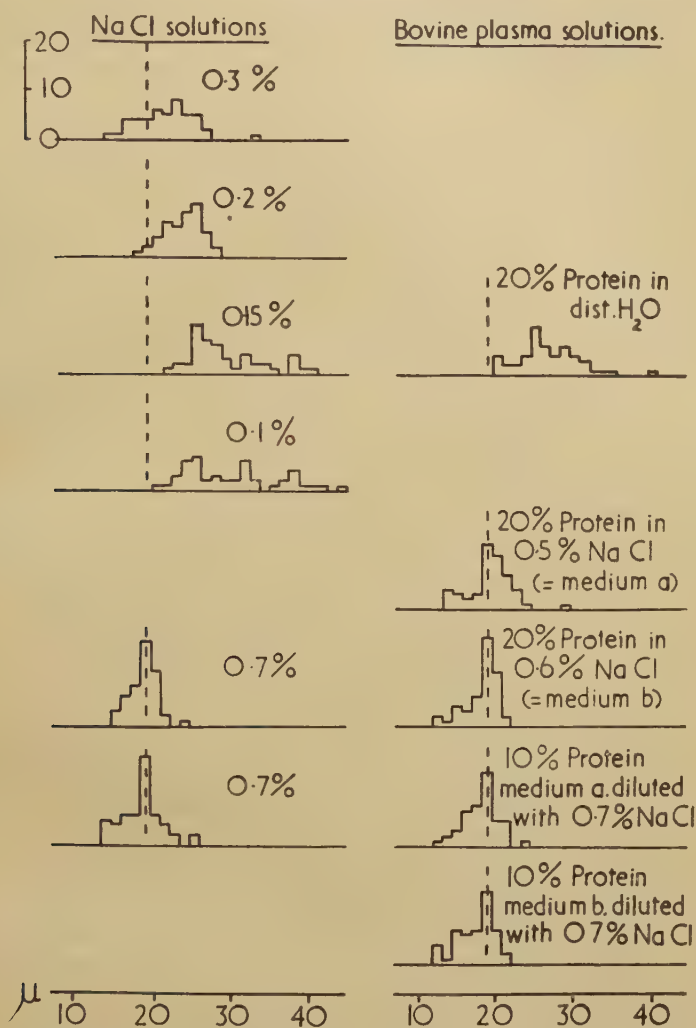


FIG. 1. Histograms showing diameters of snail spermatocytes in various media. For explanation see text. Ordinates, number of cells; abscissae, cell diameters.

These are very similar to those obtained in 0.7 per cent. protein-free sodium chloride. Each of these media was then diluted with an equal volume of 0.7 per cent. sodium chloride, giving 10 per cent. protein solutions. The histograms obtained in these media were again closely similar to those in 0.7 per cent. sodium chloride. Thus within the limits of accuracy of the method the simple rule that a 10 per cent. protein concentration is approximately

equivalent to a 0.1 per cent. sodium chloride concentration appears to be justified. A 20 per cent. protein solution is sufficiently concentrated to cause reversal of contrast in the cytoplasm of most tissue-cells. In special cases it may be necessary to use much more concentrated solutions, and here it is possible that slightly greater errors may be introduced. If, for example, a 10 per cent. solution of protein were equivalent in tonicity to 0.08 per cent. sodium chloride instead of 0.1 per cent. the error in working with a 20 per cent. protein solution dissolved in 0.5 per cent. sodium chloride would only be 0.04 per cent. of sodium chloride, assuming 0.7 per cent. sodium chloride to be isotonic. If, on the other hand, a 40 per cent. protein solution were used, dissolved in 0.3 per cent. sodium chloride, the error would be equivalent to 0.08 per cent. sodium chloride. In view of the fact that snail spermatocytes appear to maintain the same size over quite a wide range of salt concentrations, neither of these errors is likely to be of much importance with these particular cells. It may be, however, that other cells are more sensitive to salt concentration, and if in addition they require the use of concentrated protein solutions, the errors may be significant. This may be the case with mammalian red blood-cells, which are very sensitive to small changes in salt concentration and also have high refractive indices, requiring the use of protein solutions of up to 40 per cent. concentration. It is generally stated that 0.9 per cent. sodium chloride is isotonic for mammalian cells, though Ponder (1948) takes 1 per cent. as isotonic for red cells. According to the simple rule given above a solution of 40 per cent. bovine plasma albumin in 0.5 per cent. sodium chloride should be isotonic with 0.9 per cent. sodium chloride, but the values for mean corpuscular haemoglobin concentration obtained by cell refractometry with such a solution are 10–20 per cent. lower than the accepted clinical values. If a 40 per cent. protein solution dissolved in 0.6 per cent. sodium chloride is used, the results agree with the lower normal clinical values, while if 0.7 per cent. sodium chloride is used instead of 0.6 per cent., the results are in excellent agreement with clinical values. Recent freezing-point determinations by Dr. D. A. T. Dick have shown that a 10 per cent. bovine plasma albumin solution in distilled water is actually equivalent to 0.08 per cent. sodium chloride. On this basis therefore a 40 per cent. protein solution should be made up in 0.6 per cent. sodium chloride, if 0.9 per cent. sodium chloride is taken as isotonic; or in 0.7 per cent. sodium chloride, if Ponder is correct in taking 1 per cent. sodium chloride as isotonic. With the majority of cells, apart from red blood-cells, these differences are unimportant and the simple rule given above can be used.

Are individual cells capable of osmotic regulation?

Much work has been done on the osmotic regulation and water balance of whole organisms, both vertebrate and invertebrate. On the other hand, almost nothing seems to be known about the osmotic regulation of individual tissue-cells. The possibility that such cells may be capable of regulating their volume and water content to some extent at least cannot be ignored. If the

concept of the constancy of the internal environment has any general validity, it might be expected that it would be advantageous for the cell to maintain a more or less constant volume and water content, thus ensuring that the concentrations of enzymes, nucleic acids, and other substances essential for normal metabolism, growth, and cell-division should be kept within fairly well defined limits. It may be argued that in mammals at least the external environment of the cell is kept fairly constant by various mechanisms (e.g. respiratory exchanges and renal excretion), which regulate the composition of the blood. Some of these mechanisms, such as changes in renal excretion, only act slowly, however, and cells may very well be exposed to quite large temporary fluctuations in the composition of tissue fluid. Thus Hill, Long, and Lupton (1924) showed that enormous quantities of lactic acid may be liberated in severe exercise, and blood concentrations of over 100 mg. per 100 c.c. may be attained. The local changes in the immediate vicinity of the tissues may perhaps be much greater. That too much faith should not be placed in the constancy of blood composition in an individual has been shown by Schreider (1953). He carried out a statistical investigation on the reliability of a number of physiological and biochemical characteristics and showed that some of these were so variable that in order to determine a modal value, 15–20 measurements on different days might be needed for one individual. Schreider reached the disturbing conclusion that 'the postulate of fixity must be rejected on biometrical grounds'. This does not necessarily mean that the internal composition of cells varies greatly or that there is any immediate correlation between intracellular conditions and fluctuations in blood composition. Schreider himself has pointed out that living organisms contain many internal environments separated by barriers which may serve to damp out fluctuations. In terms of the present problem such barriers might constitute a regulating mechanism whereby the cell volume is maintained relatively constant in the face of quite large variations in composition of the external medium. It must be admitted that direct evidence for osmotic regulation in single cells is very scanty. The observations of Ross (1953) on snail spermatocytes and of Shapiro and Parpart (1937) on mammalian leucocytes suggest that some degree of regulation does occur over a surprisingly large range of salt concentrations. It must be remembered, however, that these workers estimated cell volumes by measuring the cell diameters with a micrometer eyepiece. It is doubtful if such measurements of diameter can be made with an accuracy greater than about 2.5 per cent., corresponding to $\pm 0.5\mu$ in a diameter of 20μ . The error in cell volume is therefore three times this amount or 7.5 per cent. Many more measurements of this type on a variety of cells would be desirable. Much evidence which may have an important bearing on this question has been assembled in a valuable review by Robinson (1953) on the active transport of water in living cells. Robinson propounds the view that the cell is in a state of dynamic equilibrium and that metabolic processes involving energy changes are necessary for maintaining a constant cell volume. The cell contents appear to have a higher osmotic pressure than

plasma and are not in thermodynamic equilibrium with it. There is an impressive body of facts to show the correlation between cell volume and respiratory processes; if the latter are depressed by the action of poisons, cellular swelling occurs in individual cells, and in whole animals the extra-cellular fluids become more concentrated. Many effects of this type are reversible. Further evidence in support of these views has been provided by

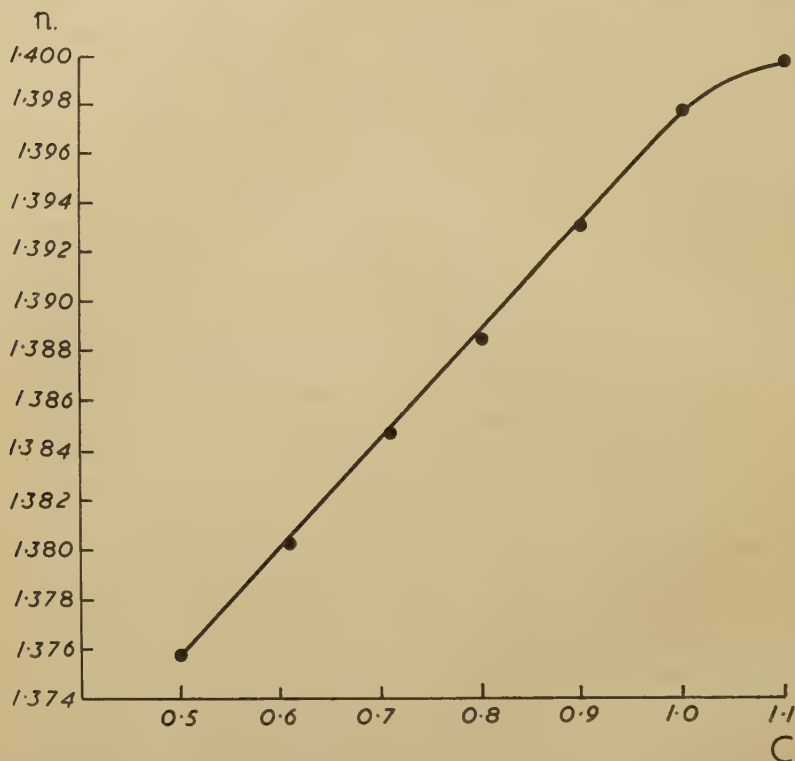


FIG. 2. Relationship between mean refractive index (n) of human red blood-cells and equivalent salt concentration of protein medium expressed in terms of grams per cent. of sodium chloride (C).

the work of Krebs and his school (for references see Bartley, Davies, and Krebs, 1954). This concept certainly fits in with observations made during the course of the present work, for example the swelling of cells found in the first stages of autolysis in sealed slide-coverslip preparations. From the point of view of immersion refractometry the existence of some degree of osmotic regulation would be extremely useful, as it would allow for some margin of error in making the immersion exactly isotonic. The presence of protein in the medium may itself be a stabilizing factor in view of Shear's (1935) finding that swelling in salt media was retarded or reversed by several proteins.

The refractometric method can itself be used to investigate this problem. As a result of swelling or shrinkage, the concentration of solids and hence

the refractive index will change; the reaction of the cell to changes in salt concentration can therefore be studied by measuring its refractive index in bovine plasma albumin solutions to which different amounts of salt have been added. Fig. 2 shows some preliminary results obtained by Miss F. M. Gaffney on human red blood-cells. The ordinates are the mean refractive indices of cells of one individual, the abscissae being the salt content of the medium expressed in terms of equivalent sodium chloride concentration. The relationship is virtually linear between 0.5 and 1.0 per cent. of salt, but there is considerable deviation from linearity above 1.0 per cent. This is only to be expected, because the cell contents can obviously only be packed down to a limited extent. Thus over the range studied there appears to be no indication of any special regulatory ability on the part of the cell. This is in keeping with our knowledge of the behaviour of red blood-cells. We have, however, carried out a few experiments on lymphocytes and have found evidence of a plateau in the region between salt concentration 0.8 per cent. and 1.0 per cent. This may suggest some degree of regulation. Further work on the problem is in progress.

EVIDENCE FOR NON-TOXICITY OF THE MEDIUM

It is extremely difficult to provide clear evidence that a medium is completely harmless to all types of cells. In the case of cells which are non-motile and which do not ordinarily undergo division, the evidence must rest mainly on the general appearance of the cell before and after immersion and on the reproducibility of refractive index measurements. Provided that mechanical damage and compression which often lead to swelling of the cell are avoided, repeated estimations of refractive index should give very reproducible results. Recently Dick (1955), working in this laboratory, has developed a simple perfusion chamber which enables cells to be irrigated with many changes of fluids of different composition. Even after more than ten changes of protein or gum acacia, cultures of chick fibroblasts or snail amoebocytes often remained in excellent condition and gave reproducible values for refractive indices.

The assessment of damage is perhaps easier in the case of motile or dividing cells. The evidence may be summarized as follows:

Protozoa

Most motile protozoa appear to tolerate bovine plasma albumin dissolved in distilled water quite well, particularly at concentrations below about 20 per cent. At higher concentrations motility decreases or may be abolished altogether. It is not always possible to decide whether this is due to a toxic action or simply to the resistance to motion offered by the viscous medium. In some cases normal motility is recovered when the organisms are transferred from a very concentrated solution to water. One cannot, however, exclude the possibility that concentrated protein solutions may exert some sort of surface action or may hinder the diffusion of gases or nutrient materials

into or out of the cell. Occasionally one comes across organisms which do not appear to tolerate the acidity (pH 5.2–5.5) of ordinary albumin solutions. Better results may be obtained in such cases by using dialysed pH-adjusted albumin.

In general, amoebae appear to be more sensitive to protein than most other Protozoa studied, but even so they remain in good condition in concentrations below about 15 per cent. for about 30 minutes. Since most amoebae are reversed in contrast in such concentrations, it is not usually necessary to use higher ones. Slightly longer survival can be obtained with pH-adjusted protein. *Euglena*, *Stylonychia*, and *Rhabdostyla* all appeared to tolerate concentrations up to about 30 per cent. of ordinary albumin and 40 per cent. or more of pH-adjusted albumin. *Chylodon* and *Podophrya* appeared in good condition in up to 40 per cent. of ordinary albumin. The parasitic forms *Trichomonas muris* and *Giardia* were strongly reversed and in good condition in 30 per cent. ordinary albumin. *Vorticella*, on the other hand, though it tolerated fairly low concentrations, usually underwent a strong contraction in high concentrations of even pH-adjusted albumin.

Ciliated epithelial cells

Scrapings from the frog respiratory passages contain ciliated cells which continued to exhibit vigorous ciliary movement for long periods in concentrations of ordinary albumin which bring about strong reversal of the cytoplasm.

Spermatozoa

The spermatozoa of a very large number of species ranging widely over the animal kingdom have been examined. In concentrations of ordinary albumin not exceeding about 10 per cent. there is scarcely any noticeable effect on sperm motility, and cells survive in apparently good condition for long periods. Human spermatozoa, for example, will survive up to about 3 days in a 5 per cent. albumin solution. In higher concentrations motility is reduced, probably because of increasing viscosity. The structural components of most sperm-heads are very dense and require high concentrations (generally between 35 and 55 per cent.) of albumin in order to bring about reversal. For this reason sperms which are reversed in contrast are usually immotile or very feebly motile. Of the various media tested, only bovine plasma albumin and dialysed egg albumin gave satisfactory results with spermatozoa.

Amoebocytes

The amoebocytes and blood-cells of several species of invertebrates have been transferred to the protein medium either directly from the blood or body fluids or from tissue cultures. Photomicrographs of an earthworm cell undergoing active movements in a protein solution sufficiently concentrated to cause strong reversal of contrast were shown in fig. 1 of the first paper of this

series (Barer and Joseph, 1954). In general, such cells appear to tolerate both ordinary and pH-adjusted albumin quite well and promising results have recently been obtained with acacia gum.

Mammalian leucocytes

Good motility has been observed in mammalian white cells kept for quite long periods in albumin solutions. A remarkable example of their hardiness was found by chance in a preparation of rat leucocytes which had been examined for several hours in a protein concentration sufficient to bring about reversal of contrast. The sealed slide-coverslip preparation was left overnight on the microscope stage at room temperature. The next morning, on glancing casually down the microscope we were astonished to find many leucocytes still undergoing active movement across the field of view.

Growth of fungal spores

Excellent germination and development of the spores of many different types of fungi has been observed even in high concentrations of albumin. The complete life-cycle from germination through the sexual and asexual phases to the formation of new spores has been followed in slide-coverslip preparations (Barer and Joseph, 1955). No nutrient substances were added to the protein medium. The ability to support the growth of fungi is in some respects a disadvantage because stock protein solutions frequently become contaminated with yeasts or moulds after about 5 days even if kept in a refrigerator. Spore germination has also been observed in acacia gum solutions, but further growth and development are slower and do not usually reach so complete a stage as in protein.

Cell division

Perhaps the strongest evidence which suggests the comparative harmlessness of bovine plasma albumin is the fact that normal cell division can be observed in it. Occasional divisions have been seen in the germ cells of the locust (Ross, 1954) and we have also observed the complete cycle in the protozoon *Cothurnia*. Such divisions are, however, comparatively infrequent and sporadic. Much more successful results have been obtained with grasshopper spermatocytes, and one can be reasonably certain of finding active division in several cells of every single preparation. Typical photographs of this process are shown in fig. 3. In some cases divisions have been followed in the same preparation for as long as 3 days. All our experiments have been carried out so far with ordinary albumin at pH 5.5 and without the addition of nutrient substances. Experiments to determine whether the rate of division can be influenced by such substances are in progress.

Regarded as a whole, the evidence quoted above, though not as complete as one could wish, nevertheless suggests that at least for a very large number of different types of cells the albumin medium and in some cases acacia gum, are comparatively innocuous.

We again wish to thank the Rockefeller Foundation, Royal Society, and Medical Research Council for making this work possible. We also wish to acknowledge the help of many colleagues, particularly Dr. D. A. T. Dick, Miss F. M. Gaffney, and Mr. K. F. A. Ross. Dr. A. G. Ogston has kindly supplied several protein samples and Dr. W. E. van Heyningen and Dr. E. Bidwell provided facilities for freezing-drying.

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FIG. 3 (plate). Photomicrographs showing first meiotic division of primary spermatocytes of the grasshopper *Chortippus parallelus*. Division is taking place in a 12 per cent. solution of bovine plasma albumin (equivalent tonicity 1.1 per cent. sodium chloride). A, diakinesis (late stage). B, diakinesis, shortly before disappearance of nuclear membrane. Note bright nucleoplasm (25 min.). C, early disruption of nuclear membrane (1 hour 25 min.). D, complete disruption of nuclear membrane. Chromosomes have moved to periphery of cell (1 hour 27 min.). E, prometaphase. Chromosomes beginning to line up on equatorial plate. Some faint spindle-fibres can be seen (2 hours 27 min.). F, prometaphase. Two dark centrosomes are visible (2 hours 40 min.). G, late prometaphase. Thick spindle-fibres visible (4 hours 30 min.). H, metaphase. Distinct spindle-fibres. Chromosomes and spindle are displaced by a crescentic mass of mitochondria (7 hours 6 min.). I, early anaphase. Chromosomes beginning to separate. Mitochondria are now clustered on each side of the spindle. Note large bright zone around chromosomes. The outline of the cell shows irregular bulging. (25 hours 47 min.). J, anaphase. The centrosome and spindle-fibres can be seen. Note also prominent polar bulging and central bright zone (26 hours 5 min.). K, advanced anaphase. Mitochondria are beginning to form 'sheaves'. Vigorous polar bulging with elongation of cell (26 hours 15 min.). L, telophase. Incomplete cleavage. Note mitochondrial sheaves, from the ends of which granules appear to be forming. Bulging in equatorial and polar axes (26 hours 40 min.). M, cleavage almost complete. Cells still linked by mitochondrial sheaves and remains of spindle. Granules at ends of sheaves more distinct (26 hours 51 min.). N, separation of daughter-cells which are still linked by a mitochondrial bridge (28 hours 13 min.). O, division complete. Daughter-cells transformed into secondary spermatocytes (48 hours 35 min.).

All photographs were taken with a Zeiss Winkel phase-contrast microscope with 4 mm. fluorite objective of N.A. 0.75. Length of scale, 25 μ .

Times from the beginning of observations are given in brackets. It should be stressed that these cells are spherical and in no way compressed. With the microscope used, similar cells in a saline medium give very poor images in which almost no internal detail can be made out. A discussion of this effect and further examples will be found in Barer, R., Naturwiss., **41**, 206 (1954).

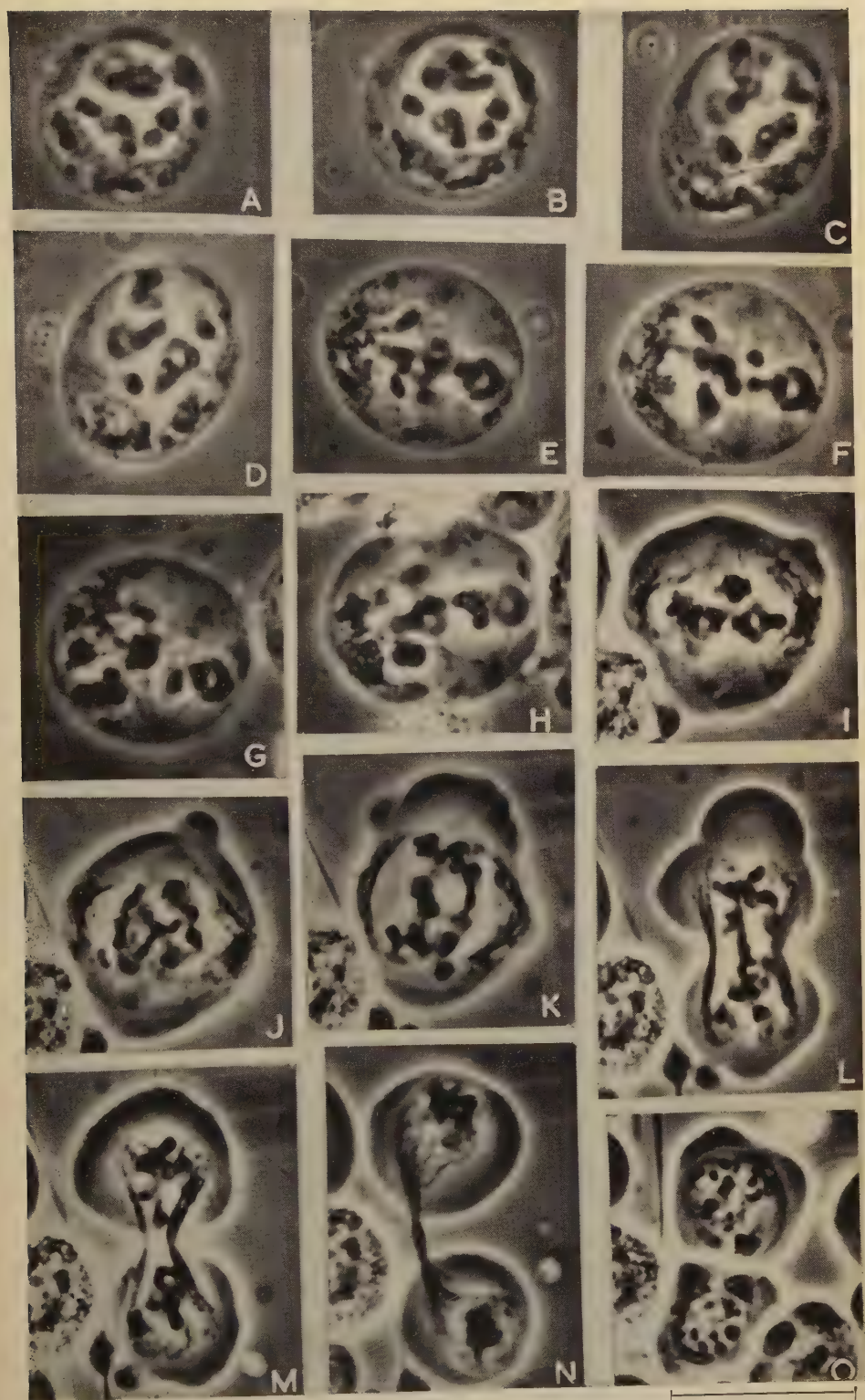


FIG. 3
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Methyl Green and Pyronin Staining of Frozen-dried Tissue

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With 2 plates (figs. 1 and 2)

SUMMARY

Frozen-dried tissue stains readily with methyl green / pyronin. The gross intracellular distribution of nucleic acids in frozen-dried pancreatic tissue stained with methyl green / pyronin corresponds with that found in chemically fixed tissue.

It has been suggested by Brachet (1953) that his methyl green / pyronin method (1940) is not readily applicable to frozen-dried material. It is the purpose of the present paper to consider this conclusion of Brachet's.

The specificity of methyl green and pyronin has been the subject of much recent work (Kurnick, 1950; Taft, 1951*a* and *b*).

OBSERVATIONS ON THE TECHNIQUE OF METHYL GREEN-PYRONIN STAINING

The following procedures were adopted as standard, and will be so described.

In all cases the material was fresh albino rat tissue.

Frozen-dried tissue

Cooled immediately in liquid propane, the latter cooled by liquid nitrogen. Dried at -40° C. for 2-4 days in a tissue-dryer employing the principle of the cold surface trap (cooled by solid carbon dioxide / alcohol mixture to about -78°) for water vapour under reduced pressure (Bell, 1952).

Infiltrated with 54° paraffin.

Sections cut at 7μ and attached to new slides by floating on a drop of acetonitrile on the slide and application of just sufficient heat to flatten.

Dried on a hot plate.

Paraffin removed and sections rehydrated through alcohols.

Stained by (a) the methyl green / pyronin technique suggested by Brachet (1953), with a solution held with acetate buffer at pH 4.7 and differentiation in 75 per cent. ethyl alcohol, or (b) the technique suggested by Taft (1951*a*) with a similarly buffered solution and differentiation in a mixture of tertiary butyl alcohol and ethyl alcohol.

Xylene; Polystyrene.

Chemically fixed tissue

Immersed immediately in Serra solution (Brachet, 1953) and fixed for 1 to $1\frac{1}{2}$ hours.

[Quarterly Journal of Microscopical Science, Vol. 96, part 1, pp. 29-33, March 1955.]

Washed in 95 per cent. alcohol.

Absolute alcohol, xylene.

Infiltrated with 54° paraffin.

Sections cut at 7 μ and attached to cleaned slides by floating on warm water with a smear of albumen-glycerine as adhesive. Dried on a hot plate.

Paraffin removed and sections re-hydrated through alcohols. Stained as for frozen-dried tissue.

Xylene; polystyrene.

After trial of the method on a number of chemically fixed tissues, pancreas was chosen as a test material because of its combination of exocrine cells, intensely staining and with a characteristic distribution of the cytoplasmic pyroninophil material, with groups of weakly staining endocrine cells. A number of specimens of frozen-dried and chemically fixed pancreas were subjected to the standard methods of preparation and staining, being stained in batches so that frozen-dried and chemically fixed slides traversed the staining and subsequent treatment together. Under these conditions, with comparison of the sections by eye under the microscope, the result was unequivocal. Staining, green to blue of the nuclear material, rose-pink to deep red in the case of nucleoli and cytoplasm, was as intense in frozen-dried as in the control (chemically fixed) pieces of tissue (fig. 1, A, B, C, D). This result was constantly obtained and was subsequently found to be uninfluenced by the staining method chosen or by all except one of a number of variations of technique now to be described. It thus appeared that there is nothing in frozen-dried tissue as such which inhibits its reactions with methyl green and pyronin.

Attempts were now made to define factors which might operate during manipulation to alter the staining capacity of frozen-dried tissue. In the course of these, some incidental conclusions were reached on the technique, and a description of them is included.

Preparation. During this stage the section of tissue in paraffin is fixed to a slide, its wax is dissolved out, and it is re-hydrated. Until this stage it may be expected that a considerable proportion of the protein in frozen-dried material will remain to be denatured, but that the action of heat and alcohols will bring about further denaturation. Before this occurs, however, the cells are relatively susceptible to loss of protein substances soluble in water, including stainable nucleoproteins. Tissue exposed to water at this stage may be expected to lose staining capacity. It is possible that atmospheric water vapour might be a factor, but a more potent one would be an aqueous solution such as the egg-albumen used as a section adhesive.

Frozen-dried paraffin sections (a) stuck to slides by the standard method were compared with sections (b) pressed on to a slide, the surface of which was smeared with albumen-glycerine adhesive and with sections (c) floated on a drop of distilled water on the slide and subjected to heat sufficient to flatten. The slides, after attachment of the sections, were all dried on a hot plate and subjected to the standard rehydration, staining, dehydration, and

mounting procedures. If the sections were on a thick layer of albumen-glycerine such as might require over an hour to dry on the hot plate, the tissue spread and disintegrated; with thinner films of adhesive and with water, drying out quickly, sections were obtained which could be stained. Compared with the controls (*a*) these sections all showed spreading and various grades of reduction in staining capacity. In the case of slides floated on water, staining was minimal, although differentiation between pyronin and methyl green staining components of the cell was still recognizable (fig. 2, C, D).

Other possible mechanisms for this effect are that the tissue itself or the egg-albumen solution may contain nucleases, whose action would be favoured by hydration.

Staining. Satisfactory staining was obtained with both dyes, whichever of the standard staining procedures was used. It is known that results depend upon the quality of the dyes. The methyl green of this series was a Revector Microscopic Stain produced by Hopkins and Williams, and this stained satisfactorily. Two pyronins were tried, of which one stained feebly, particularly when used according to the procedure of Brachet. The other, produced by Geigy, gave very satisfactory results. This finding was confirmed by testing comparable sections in staining solutions differing only in the brand of pyronin used. Intensity of pyronin staining differed considerably.

Dehydration and mounting. The well-known lability of pyronin in tissue during differentiation with alcohol was observed here in the use of the original pyronin. In this instance the method of staining and dehydration with tertiary butyl alcohol / ethyl alcohol mixture as recommended by Taft was found to give the more stable staining and the greater final intensity. With the methyl green of Hopkins and Williams, the pyronin of Geigy, and the staining solutions of Brachet and Taft, staining was stable and, in fact, the stained tissue could be subjected to differentiation for 10 minutes in 70 per cent., 95 per cent., or absolute ethyl alcohol without excessive loss of pyronin.

Since differences in reaction to alcohol during differentiation might form the basis of differences of staining, pairs of slides, one in each pair fixed in Serra's fluid, the other frozen-dried, were subjected to differentiation for 10 minutes in varied strengths of alcohol from 70 per cent. to absolute. No differences were observed.

INTRACELLULAR DISTRIBUTION OF STAINABLE SUBSTANCES

The distribution of stainable substances in pancreatic exocrine cells of the rat, frozen-dried, is shown in fig. 1, A, B. The regularly outlined nuclei stain diffusely green, and through this background more intensely green-staining material is dispersed in irregular masses and particularly concentrated in a zone immediately surrounding the nucleolus. There is also a thin zone of deeper green-staining in the region of the nuclear membrane. The nucleoli, of which there is one or more in each nucleus, stain a clear rose-pink. The cytoplasm of these cells contains pyronin-staining material with a distinctly

zonal distribution. In a region that is variable in width but comprises about one-half to two-thirds of the depth of the cell from the internal cell margin bounding the gland lumen, the pyronin is distributed in an approximately reticular pattern, appearing to occupy space between large granules of non-staining substance. In an external region extending to the outer border of the cell, the pyronin-stained material is confluent and deep red, and this region usually completely contains the nucleus or the latter may project partially into the internal region.

The distribution of these substances in pancreatic exocrine cells fixed in Serra's solution is shown in fig. 1, C, D. It conforms grossly to that within frozen-dried cells though the latter appear better preserved. Compared with the frozen-dried cells there appears to be some shift of cytoplasmic substance (compare Bell, 1952); but, since the distribution may depend on the functional state of the gland cell, no conclusions on its relation to chemical fixation will be drawn from this material.

The cytoplasm of the frozen-dried pancreatic endocrine cells stains palely throughout without regional variation, the nucleus also palely, the nucleolus relatively deeply (fig. 2, A, B).

DISCUSSION

When the routine technique described here is used, frozen-dried tissue is suitable for the application of methyl green / pyronin staining reactions. Further, the result is reliable and the staining is stable and not disturbed by variations of differentiation technique. The frozen-dried and chemically fixed tissues seem to behave in the same way during staining and to the eye have approximately the same intensity of colour. Taft also reported satisfactory results (1951a). To understand why some workers have failed to stain frozen-dried tissue, it is necessary to seek factors which may contribute to differences between its behaviour and that of chemically fixed tissue. One such is the action of water. If sections are floated on water as a preparation for alcoholic dehydration, a striking difference in staining behaviour results (compare figs 1, A, B and 2, C, D). Contact with water at this stage has no effect on the subsequent reaction in the case of chemically fixed tissue but may almost abolish it in the case of frozen-dried tissue. This effect might be due to the diffusibility of the frozen-dried substances or possibly to their susceptibility to nucleases which may be present. Another possible action which requires consideration, in view of the suggestion of Brachet (1953) that lipides retained in frozen-dried material may influence the course of the staining

FIG. 1 (plate). A and B, exocrine cells of rat pancreas, frozen-dried and stained with methyl green / pyronin. A shows the distribution of pyronin-staining (internal and external cytoplasm, nucleoli). B shows distribution of methyl green (nuclear periphery and round the nucleoli).

C and D, exocrine cells of rat pancreas, fixed with Serra and stained with methyl green / pyronin. C shows the distribution of pyronin staining; D that of methyl green. Compare with A and B.

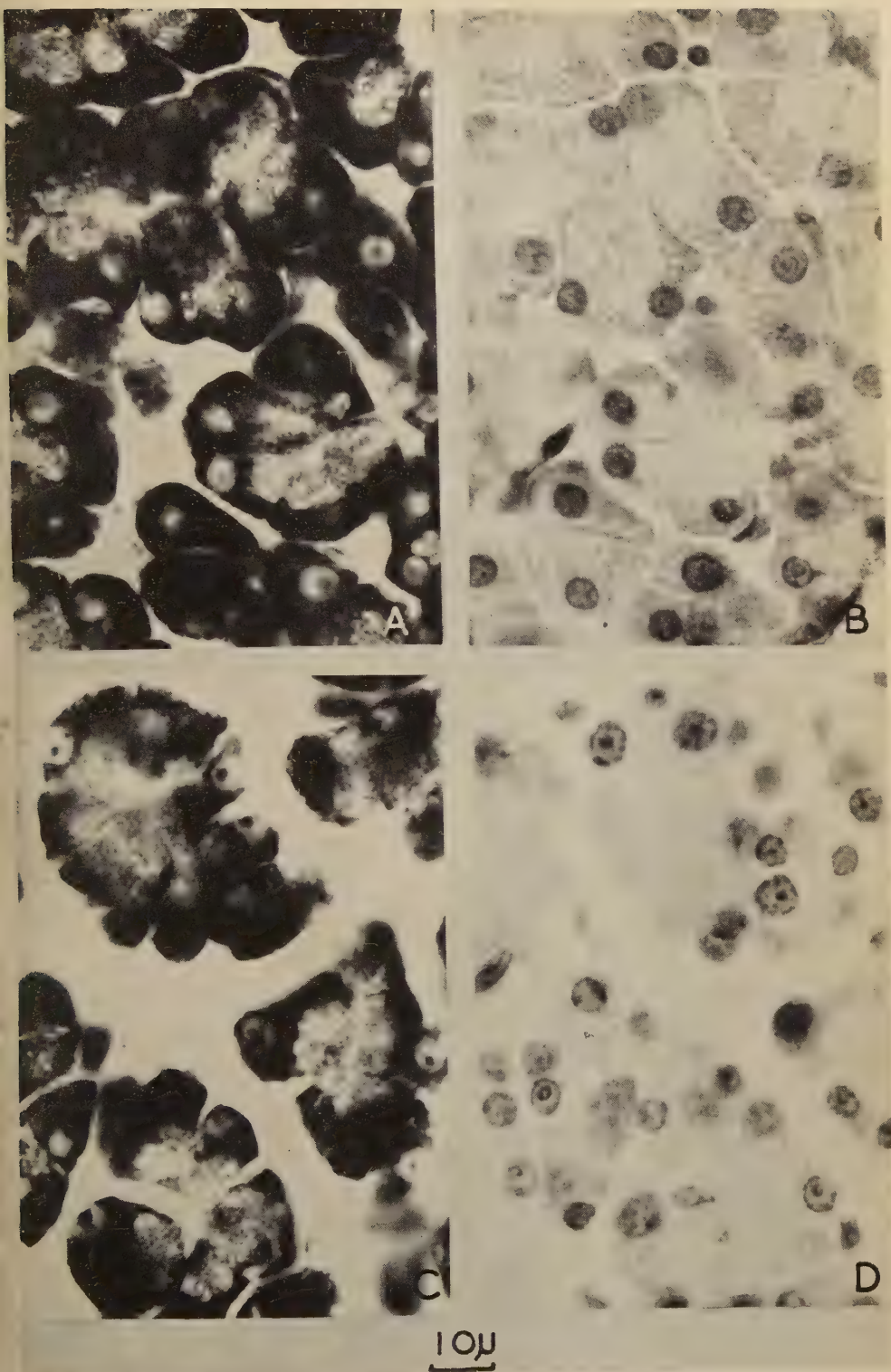
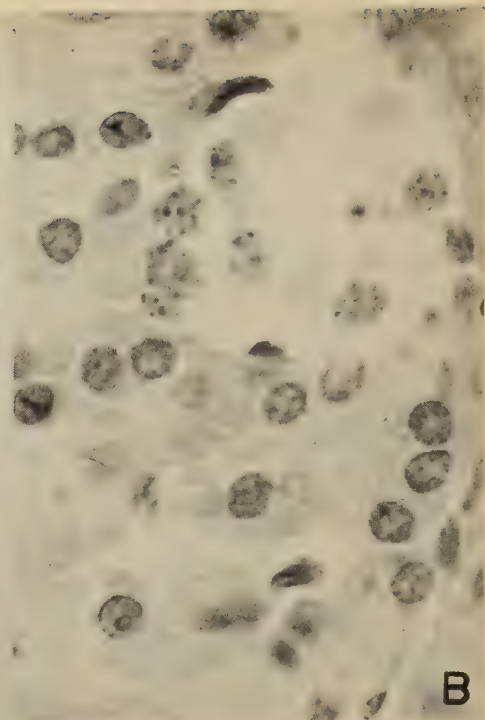


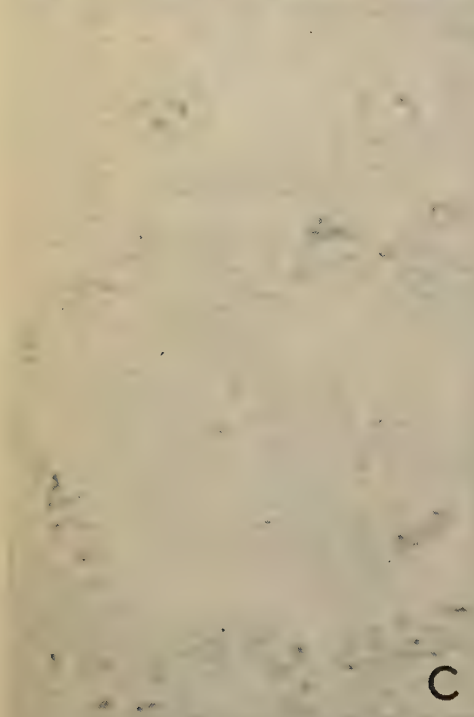
FIG. 1
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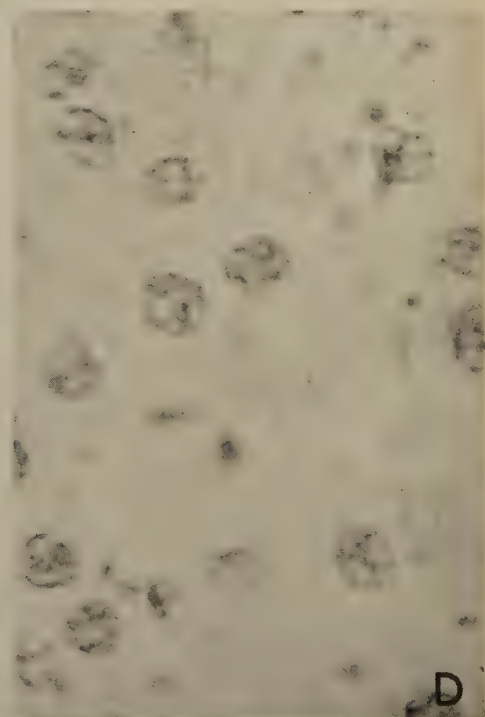
A



B



C



D

10μ

FIG. 2
J. O. LAVARACK

reaction, is one of solution of these lipides by acetonitrile used in attaching the sections. Though it may occur, removal of lipides in this way is apparently not a prerequisite for the staining, since equally good results are obtained if the sections are simply attached to the slide by heat.

There is general agreement that the nucleoproteins are included in the basiphil substances of the cell and this view is based on the Feulgen aldehyde reaction, the action of nucleases, and absorption measurements. Localization of these nucleoproteins in cells requires careful consideration of the conditions under which they were observed. The Feulgen-positive and the basiphil substances of pancreatic cells have been plotted in chemically fixed and in frozen-dried material. Chemically fixed material has also been used for observations on basiphil substances combined with selective destruction by nucleases (Brachet, 1940). Absorption measurements have been made on frozen-dried material, in this case unstained and simply transferred through paraffin to glycerine (Caspersson, 1950). That deoxyribose nucleic acid is distributed in the nucleus and ribose nucleic acid in the nucleolus and cytoplasm and that there are cytoplasmic zones of ribose nucleic acid are conclusions derived from observation on cells, whether frozen-dried or chemically fixed, stained or unstained. So far, therefore, although the evidence is not exhaustive, it is consistent with a supposition that this is the distribution of nucleic acids during life.

It is a pleasure to thank Prof. J. F. Danielli and Dr. L. G. E. Bell for much instruction and help in many ways during my stay in the Department of Zoology, King's College, Prof. J. Brachet for his kindness and decisive assistance during a short visit to his department, and Mr. A. T. Green for the microphotographs. The work was done during the tenure of a C. J. Martin Fellowship of the Department of Health, Australia. The assistance of the Department is gratefully acknowledged.

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FIG. 2 (plate). A and B, islet of endocrine cells of rat pancreas, frozen-dried and stained with methyl green / pyronin. A shows the distribution of pyronin staining, B that of methyl green.

C and D, exocrine cells of rat pancreas. The photomicrographs show the effect of exposure to water after freezing-drying, with subsequent staining by pyronin (C) and methyl green (D).

The Histochemical Localization of β -Glucuronidase in the Digestive Gland of the Roman Snail (*Helix pomatia*)

By F. BILLET T AND S. M. MCGEE-RUSSELL

From the Department of Zoology and Comparative Anatomy, University Museum, Oxford)

With one plate (fig. 4)

SUMMARY

A modification of the histochemical technique for the localization of β -glucuronidase originally suggested by Friedenwald and Becker (1948) has been applied to the digestive gland of the gastropod *Helix pomatia*. In the original technique the ferric 8-hydroxyquinoline formed by the enzymic hydrolysis of quinolyl-8-glucuronide, in a saturated solution of ferric 8-hydroxyquinoline, was converted to Prussian blue. The Prussian blue conversion is omitted in the technique described in this paper as it appears to introduce errors in localization. The ferric 8-hydroxyquinoline crystals are sufficiently characteristic to be used as the end-point of the technique. The results obtained suggest that β -glucuronidase is confined to the digestive cells in the digestive gland of the snail, and is associated with secretory granules in them.

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INTRODUCTION

FISHMAN (1950) has reviewed the properties of β -glucuronidase, an enzyme which catalyses the hydrolysis of β -glucuronides. The enzyme appears to be particularly active in the kidney, liver, and spleen of mammals (Mills, 1946). Far greater activity is displayed by the crop fluid of locusts (Robinson, Smith, and Williams, 1953), and by the digestive glands of certain

gastropod molluscs (Utusi, Huzi, Matumoto, and Nagaoka, 1949; Dodgson, Lewis, and Spencer, 1953). In these invertebrates the enzyme may play some part in the digestion of plant material; a similar function may be attributed to the β -glucuronidase said to be produced by micro-organisms in the digestive systems of ruminants (Karunairatnam and Levvy, 1950; Marsh, Alexander, and Levvy, 1952). The function of the enzyme in mammalian tissues is not clear; it has been implicated in growth and regeneration processes (Levy, Kerr, and Campbell, 1948), and in the metabolism of sex hormones (Fishman, 1947; Fishman, Riotton, Farmelant, and Homburger, 1952).

Several methods have been suggested for the histochemical localization of β -glucuronidase in tissues. The method originally suggested by Friedenwald and Becker (1948) appears to be the most satisfactory. A modified form of this technique has been developed by Burton and Pearse (1952).

Preliminary experiments upon the kidney, liver, and spleen of the mouse suggested that certain features of the technique required investigation. It was decided, therefore, to examine the technique critically, stage by stage, using a tissue known to possess a very high β -glucuronidase activity, the digestive gland of the Roman snail, as a test object for localization (Billett, 1954). It was also of interest to establish the intracellular distribution of the enzyme in the gland, in view of the possible association between β -glucuronidase and mitochondria suggested by Campbell (1949), and the probable significance of the enzyme in the digestive processes of the animal.

MATERIAL

Animals. The *Helix pomatia* were collected from a colony in a mixed wood at Seven Springs Hill, Cheltenham, and were maintained in the laboratory in damp compartments with earth floors, at room temperature, on a diet of cabbage. Animals in temporary aestivation were not used for the experiments, but in a number of instances the active animals were starved for a time before dissection.

Chemicals. Quinolyl-8-glucuronide was prepared according to the method of Robinson and others (1953). All other chemicals employed were of 'Analar' or 'Reagent' grade. Chemically clean glassware was used throughout the procedures described in this paper.

Dissection. The animal was killed by decapitation and removed from the shell. As shown in fig. 1, the digestive gland is divided into four lobes occupying two regions, A and B. One lobe, the upper, occupies the extreme tip of the helical shell and is closely associated with the ovotestis; the other three lobes overlie the principle viscera and are separated by the looped intestine which runs through the outer part of the gland. It is difficult to dissect out region A uncontaminated with ovotestis, and except in one or two experiments in which the ovotestis was deliberately included in the test material, region B alone was used. It is easy to remove both the intestine and the mantle epithelium from this region and obtain the digestive gland tissue alone.

OUTLINE OF THE PREVIOUS METHODS

In the original method of Friedenwald and Becker (1948) the enzyme in fresh tissue sections was allowed to act upon quinolyl-8-glucuronide in the presence of ferric 8-hydroxyquinoline. Deposits of the latter compound formed at the presumed site of the enzyme, were converted into Prussian blue.

Seligman and others (1951) found that the β -glucuronidase of rat liver

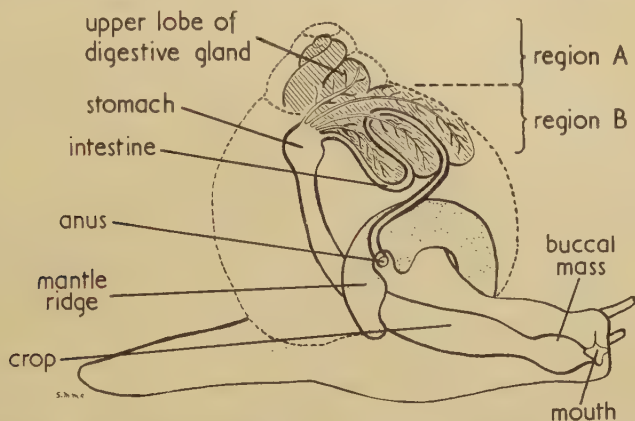


FIG. 1. A diagram illustrating the disposition of the digestive gland in the snail *Helix pomatia*.

withstood formalin fixation at about 4° C. Fixation under these conditions was used by Burton and Pearse (1952) in their modification of the original technique. This enabled these workers to cut thinner sections and to obtain better localization of the Prussian blue deposits. The essentials of the method described by these authors are as follows:

- (1) Fix tissue (rat kidney) in 10 per cent. formalin for 4–48 hours.
- (2) Cut frozen sections (10–15 μ) into ice-cold 0.1 M acetate buffer at pH 5.2.
- (3) Transfer the sections to a mixture of quinolyl-8-glucuronide, ferric 8-hydroxyquinoline, and about 0.1 M acetate at pH 5.2.
- (4) After 5–24 hours' incubation at 37° C. wash in water and transfer to albumenized slides.
- (5) Wash for 15 minutes in 0.5 M oxalate buffer (pH 4.0).
- (6) Treat with an acid solution of potassium ferrocyanide to form Prussian blue, and wash in water.
- (7) Counterstain with Mayer's carmalum, or with neutral red. Mount in DPX after dehydration in alcohols and clearing in xylene.

This technique gave disappointing results when applied to the digestive gland of *Helix pomatia*. The Prussian blue deposits were poorly localized and very much less in amount than the high activity of the tissue would lead one to expect. Examination of successful preparations in mouse tissue showed that

the crystalline precipitate of ferric 8-hydroxyquinoline was sufficiently characteristic to be used as the end-point of the technique; this appeared to be important, for upon practical and theoretical grounds the conversion to Prussian blue seemed to be a stage likely to introduce errors in cytological localization.

FIXATION

Seligman and others (1951) found that well over 80 per cent. of the β -glucuronidase activity of rat liver remained in the tissue after it had been fixed in 10 per cent. formalin, buffered to pH 7 with phosphate, at about 4° C. for 24 hours. For rat kidney Burton and Pearse (1952) found the optimum time of fixation for good histochemical localization to be 4–12 hours in 10 per cent. formalin at about 4° C.

By a method previously described (Billett, 1954) a number of determinations were made of the β -glucuronidase activity of the snail digestive gland before and after fixation in ice-cold formaldehyde-saline. This showed that after 3 hours' fixation the degree of inactivation of the enzyme varied considerably, ranging from 40 to 80 per cent. Measurements of the activity after more prolonged fixation suggested that the enzyme is at first rapidly inactivated (1–3 hours) and then more slowly (3–12 hours). Thus the enzyme in the snail digestive gland appears to be more sensitive to fixation than the enzyme in rat kidney and liver. Fixation is, however, necessary, for it is impossible to cut thin sections of the freshly frozen gland satisfactorily. At least 3 hours in ice-cold formaldehyde-saline is necessary to permit good sectioning at 20 μ .

THE PREPARATION AND PROPERTIES OF THE SUBSTRATE MIXTURE

The method of preparation recommended by Burton and Pearse is as follows. Mix 13 ml. of 0.01 M quinolyl-8-glucuronide in 0.1 M acetate buffer (pH 5.2), 13 ml. of 0.01 M 8-hydroxyquinoline in 0.1 M acetate buffer (pH 5.2), 9 ml. of 0.03 M ferric sulphate and 1.0 ml. of M acetate (pH 5.2). Incubate the mixture for at least 2 hours at 37° C., centrifuge, and store the supernatant fluid in a refrigerator.

8-Hydroxyquinoline and its glucuronide are not very soluble in water and in order to achieve a 0.01 M solution of these substances it is necessary to apply heat. On cooling, crystals re-form in the solutions, particularly if these are placed in a refrigerator. This difficulty was overcome by dissolving the quinoline and its glucuronide in the same solution of deci-molar acetate buffer, at the required pH, to make a 0.005 M solution with respect to each substance (e.g. 14.5 mg. 8-hydroxyquinoline and 37.5 mg. of glucuronide in 20 ml. of acetate buffer). In this way a clear solution which does not crystallize was obtained and the danger of having a reduced concentration of the substrate avoided. Alternatively, the compounds may be dissolved together in water and the requisite amount of molar acetate buffer added.

Two reactions appear to occur when the substrate mixture is prepared.

First, the ferric sulphate reacts with the 8-hydroxyquinoline; this gives the mixture a green colour and results in formation of a precipitate. Secondly, the ferric sulphate reacts with the acetate forming a precipitate of basic ferric acetate. Table 1 shows the effect of pH on these reactions. Clearly the properties of the substrate mixture depend to a certain extent on the pH. Below 4.5 (added acetate) there is a sudden intensification of the green colour, presumably due to an increased concentration of ferric 8-hydroxyquinoline in solution.

TABLE I
The effect of pH on the substrate mixture

<i>pH of added acetate</i>	<i>pH after 2 hours' incubation</i>	<i>Colour of mixture</i>	<i>Precipitate</i>	<i>Effect of acetate on $Fe_2(SO_4)_3$ alone</i>
3.56	2.46	Very dark green	None	No precipitate
4.00	3.06	Very dark green	Slight	Precipitate
4.46	3.85	Dark green	Heavy	Heavy precipitate
4.85	4.26	Light green	Heavy	Heavy precipitate
5.20	4.58	Pale yellow-green	Heavy	Heavy precipitate

Table 1 also shows that a marked drop of pH occurs during the preparation of the substrate. For the snail digestive gland it was necessary to prepare a substrate mixture close to the optimum pH of the snail enzyme; this is 4.0–4.2. The pH of the added buffer was therefore 4.5.

Apart from the modifications which have been described, the substrate mixture was prepared according to the procedure of Burton and Pearse.

It was observed that if a large number of sections was added to the substrate solution the colour of the solution became appreciably lighter. This suggests that adsorption of ferric 8-hydroxyquinoline occurs. The degree of adsorption appears to depend upon the tissue; for instance, it is greater for the snail digestive gland than it is for mouse kidney. Wigglesworth (1952) has commented on the considerable capacity of tissue sections for taking up iron from solutions of salts.

The addition of potassium hydrogen saccharate in the concentrations necessary to inhibit the enzyme also reduces the colour of the substrate mixture. This is presumably due to the formation of iron saccharate. This reaction would reduce the concentration of ferric 8-hydroxyquinoline in the substrate mixture and the concentration of saccharate ions in the control.

THE FORMATION AND PROPERTIES OF FERRIC 8-HYDROXYQUINOLINE

These were studied in the following way. 20 μ sections of formalin-fixed tissue were cut into ice-cold saline. Within 10–15 minutes these sections were

placed in an incubator at 37° C. in the substrate mixture contained in small (2-inch) Petri dishes, or in 1-inch specimen tubes. The sections were mounted in distilled water on slides at hourly intervals for observation.

Results. After incubation times varying from 1 hour to 12, crystals appeared in the test sections. Control sections, inhibited by saccharate, showed no development of crystals. In both test and control sections there was a general darkening of the tissue associated, probably, with an uptake of iron from the substrate. This darkening of the tissue was greater in the test than in the control but it did not appear to be directly related to the subsequent development of the crystalline deposits.

The size, shape, and abundance of the crystals formed varied, apparently

TABLE 2

<i>Reagent</i>	<i>Observation</i>	<i>Conclusion</i>
Distilled water (about 19° C.)	Appearance of the crystals remains unchanged for at least 7 days	Insoluble
Distilled water (about 60° C.)	Crystals dissolve in 4-5 hours	Soluble
Oxalate buffer (pH 4.0)	Crystals remain unchanged for at least 24 hours	Insoluble
0.1 M acetate buffer (pH 4.5)	Crystals remain unchanged for at least 24 hours	Insoluble
Ethanol	Some crystals dissolve in 5 minutes, most have disappeared within 30 minutes	Fairly soluble
Acetone	Crystals dissolve within 30 seconds	Very soluble
Glycerol	Crystals unchanged after 7 days	Insoluble

in accordance with a number of factors including the initial activity of the tissue, the nature of the substrate (governed by the care taken in its preparation), and the time of incubation. Under optimum conditions a rapid decomposition of the substrate occurs and results in the intracellular production of small crystals of ferric 8-hydroxyquinoline after 1-2 hours' incubation. These crystals are irregularly rounded bodies about 1-3 μ in diameter. The deposits grow rapidly with continued incubation, and after 4-5 hours are exceedingly heavy. If conditions favour slow decomposition of the substrate the initial crystal size tends to be large, the crystals are relatively few in number, and they appear to be formed irrespective of cell boundaries. Crystals formed in this way are needle-shaped (about 10 μ long), short rods (about 5 μ), and hexagonal plates (3-4 μ in diameter). Whatever their shape and size, the crystals are characteristically brown and anisotropic.

We examined the solubility of the crystals formed within the sections of the tissue in a number of solvents. Sections were transferred to solid watch glasses containing the solvent and the condition of the crystals was followed by using a low-power microscope. The observations are summarized in table 2. The rate of dissolution of the crystals is probably influenced by such

factors as their size and their site of formation in the tissue. Attention was also paid to the reaction of the crystals with the reagents employed to convert them to Prussian blue in the techniques of the previous authors. These observations are summarized in table 3.

TABLE 3

<i>Reagent</i>	<i>Observation</i>
Equal volumes of 1 N HCl and 1 per cent. (w/v) $K_4Fe(CN)_6$	Conversion of crystal deposits is incomplete after 30 minutes. Colour formed is diffuse in distribution. There is some fragmentation of the crystals
Equal volumes of 1 N HCl and 1 per cent. (w/v) $K_4Fe(CN)_6$ at 60° C.	Complete conversion to Prussian blue in 15 minutes. Colour is diffuse
Equal volumes of 1 N HCl and 2 per cent. (w/v) $K_4Fe(CN)_6$	Conversion to Prussian blue is incomplete after 30 minutes
Equal volumes of 2 N HCl and 1 per cent. (w/v) $K_4Fe(CN)_6$	Conversion to Prussian blue is incomplete after 30 minutes
1 per cent. (w/v) $K_4Fe(CN)_6$	Crystals appear unchanged after 24 hours
1 N HCl	Little change in the appearance of the crystals after 1 hour. Crystals disappear after 12 hours
1 per cent. (w/v) $K_4Fe(CN)_6$ at 60° C.	Most crystals dissolve in 4-5 hours. (Solubility in hot water)
1 N HCl at 60° C.	Crystals dissolve within 15 minutes

THE USE OF OXALATE BUFFER

After the formation of ferric 8-hydroxyquinoline, Burton and Pearse recommend washing the sections in half-molar oxalate buffer (pH 4.0) for 15 minutes. In table 2 it may be seen that prolonged washing in the buffer has no appreciable effect on the crystals. The effect of the oxalate washing is said to be the removal of ferric hydroxide, and it is noticeable that the treatment removes the general dark background coloration previously mentioned. Sections are very much 'cleaner' and more transparent after washing for an hour or longer in the buffer.

THE CONVERSION OF FERRIC 8-HYDROXYQUINOLINE TO PRUSSIAN BLUE

Ferric 8-hydroxyquinoline may be converted into Prussian blue by the action of an acid solution of potassium ferrocyanide. Burton and Pearse used a mixture of equal volumes of 1 N hydrochloric acid and 1 per cent. (w/v) potassium ferrocyanide, applied to the sections for 15 minutes. The reaction probably occurs in two stages: the acid breaks down the ferric 8-hydroxyquinoline, and the ferrocyanide reacts with the liberated iron.

The conversion of the crystals formed in our technique was readily observed under the microscope. Twenty μ sections were incubated in the substrate mixture until heavy deposits of crystals had formed. After being washed in oxalate and rinsed in distilled water these sections were subjected

to the ferrocyanide hydrochloric acid mixture, and to the separate components of this reagent. The reactions were observed with the sections in solid watch glasses, or mounted on slides beneath coverslips. The effects of a number of factors were studied. The results show that the acid ferrocyanide mixture effects a relatively slow conversion of the ferric 8-hydroxyquinoline into Prussian blue. The rate of conversion can be accelerated by using higher concentrations of acid and ferrocyanide, and by carrying out the conversion at 60° C. However, at the higher temperature the errors in localization liable

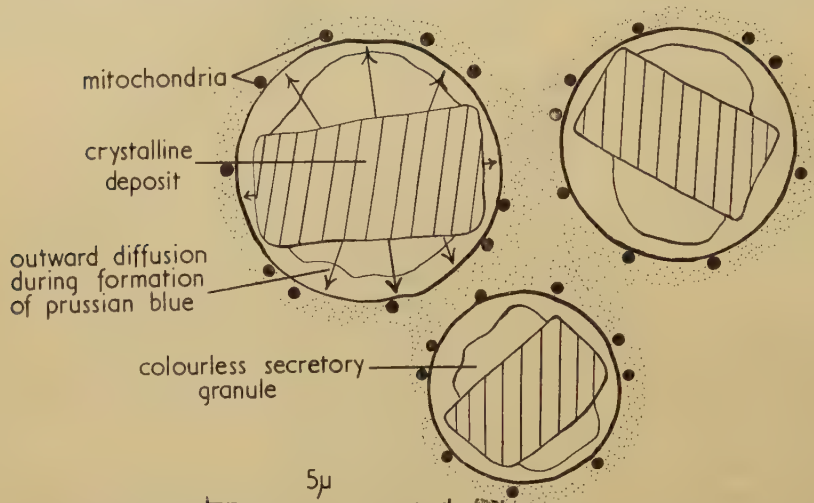


FIG. 2. A diagrammatic representation of the appearance of crystals of ferric 8-hydroxyquinoline deposited within cytoplasmic vacuoles of digestive cells in association with secretory granules.

to occur because of diffusion are likely to be greater than at the lower (laboratory) temperature. The Prussian blue deposits appear to bear only a general resemblance to the localization of the crystals, the site of which is surrounded by a diffuse blue colour. Apparently Prussian blue is as easily taken up by the tissues as iron. Observation of the conversion of single crystals with a 2 mm. oil-immersion objective shows that there is considerable diffusion of the blue colour away from the decomposing crystals; the colour is taken up heavily on the elements of the surrounding tissue. Thus for a crystal embedded in the tissue, apparently within one of the cytoplasmic vacuoles, the situation illustrated in fig. 2 results. The Prussian blue formed diffuses outwards from the crystal and colours the wall of the vacuole and any granular material which is in the vacuole. Despite repeated observations, we have been unable to ascertain whether the crystalline material is deposited around or merely close to the granular material in the vacuoles.

Thus the ferrocyanide treatment may give rise to two sources of error. First, during the conversion of the crystals to Prussian blue, considerable redistribution by diffusion may occur. Secondly, owing to the slow rate of

conversion, both Prussian blue and ferric 8-hydroxyquinoline may be present when the section is carried through the mounting procedure. Any such unconverted ferric 8-hydroxyquinoline will be rapidly removed by alcohol (table 2). There is no reason to suppose that the conversion to Prussian blue begins first at sites of highest activity, where the greatest number of crystals occur: in fact the microscopical observations suggest rather the contrary. Thus considerable misinterpretation may arise through the use of acid ferrocyanide, and it appears both desirable and practical to dispense with this treatment altogether. Modified in the way described, the technique also avoids the use of alcoholic dehydration, the sections being mounted directly in an aqueous medium. This also appears to be advantageous, for when sections of the digestive gland are passed from water into alcohol they undergo a marked visible shrinkage and distortion. Such an effect may also give a false disposition of the Prussian blue deposits, or of any crystals which survive the alcohol treatment. In the modified technique, which is given in detail below, the acid ferrocyanide treatment is omitted; we consider that the ferric 8-hydroxyquinoline crystals alone are sufficiently identifiable, and indicative of the enzyme site, provided that rapid precipitation and small crystal size are achieved.

CONTROLS

In the experiments described in this paper the controls contained potassium hydrogen saccharate, added to the substrate mixture so as to give a final concentration of the inhibitor of 0.0005 M. This concentration completely inhibits the reaction occurring in the tests with the snail tissue. Additional controls were made as follows. In some cases sections were incubated in substrate which did not contain quinolyl 8-glucuronide, in others sections were heated to 80°–100° C. and then incubated in the substrate mixture under normal conditions.

COUNTERSTAINING

A wide range of stains was tested, the majority of which were unsuitable because of their solubility in glycerol mounting media. Satisfactory staining was achieved with nuclear fast red (G. Gurr Ltd., batch no. 3569) for about 1 hour; or Mayer's haemalum for 5 minutes, followed by blueing in tap water; or methyl green/acetic for 24 hours. The acidity of the last stain did not affect the crystals.

SCHEDULE FOR THE MODIFIED TECHNIQUE

The following schedule was adopted in the light of the observations described and discussed above. Throughout the procedure the sections are handled with glass needles.

- (1) Kill a snail by decapitation and remove the digestive gland.
- (2) Place the tissue in ice-cold formaldehyde-saline and leave it in the refrigerator for 3 hours.

- (3) Wash tissue in ice-cold saline (0.9 per cent. w/v) for 20 minutes (three changes).
- (4) Cut sections at 20μ on a freezing microtome into chilled saline.
- (5) Place sections into freshly prepared substrate mixture:
test: 2.0 ml. substrate mixture with 0.1 ml. distilled water;
control: 2.0 ml. substrate mixture with 0.1 ml. 0.01 M potassium hydrogen saccharate.
- (6) Examine the sections hourly by removing and mounting one on a slide (float it on with distilled water), until heavy deposits of crystals are formed.
- (7) Rinse the sections in distilled water and wash in oxalate buffer for 1 hour.
- (8) Rinse again in distilled water and counterstain.
- (9) Float sections on to glass slides with distilled water and mount in Farrant's medium.

RESULTS

The results of this investigation may be considered under two headings: first, the findings with respect to the bases of the technique, and secondly, the results of the technique upon the selected tissue, the digestive gland of the snail. The former have been largely set out above; the latter involves some account of the structure of the digestive gland.

The histology of the digestive gland. The results of a detailed study of the histology of this tissue will be summarized shortly; a longer account is in preparation.

The digestive gland of the snail is a diverticulum of the gut, composed of a highly modified secretory epithelium grouped into blind-ended tubules. The tubules are loosely bound together by connective tissue, and by a ramifying system of haemocoelic ducts. Histologically the organ is complex, but it shows no differentiation into locally specialized areas, the numerous cell-types occurring everywhere throughout the tissue.

Previous authors have distinguished three principal cell types in the secretory epithelium: the *Kalkzellen* or calcium cells, which are the most characteristic; the *Fermentzellen*, *Keulenzellen*, or ferment cells; and the *Leberzellen*, *Körnerzellen*, or liver cells (Barfurth, 1883; Frenzel, 1885; Cuenot, 1892). More recent authors (Wagge, 1951; Fretter, 1953) have not distinguished between the last two types named. Fretter (personal communication) considers that the digestive cells may be regarded as having both secretory and excretory functions, and we agree with this view. Krijgsman (1925; 1928) detected a secretory cycle in both the salivary glands and the digestive gland. He also distinguished only calcium cells and digestive cells in the digestive gland epithelium. We are in substantial agreement with this point of view, but would point out that the category denoted by 'digestive cell' includes a number of cell-types of different form, including those distinguished by earlier authors as *Fermentzellen* and *Leberzellen*, which probably

represent stages in two principal cycles of excretion and secretion (McGee-Russell, in preparation). Fig. 3 illustrates the four forms of cell which we can detect in the digestive epithelium fixed by this technique: three (A, B₁, and B₂) are digestive cells in what are presumed to be different stages of cell activity; the fourth is the characteristic calcium cell (C).

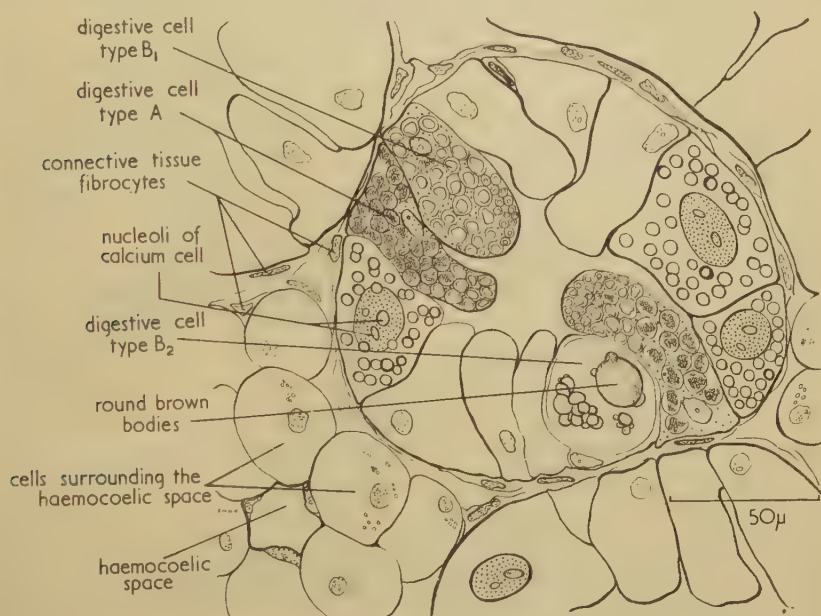


FIG. 3. A diagram of the structure of the digestive gland tubules of *Helix pomatia* cut in transverse section.

The digestive cells are all elongate club-shaped or rounded cells varying in height from 50 to 100 μ in fixed preparations. There is a basal nucleus about 5 to 10 μ in diameter, usually with a single nucleolus which is not prominent. The cytoplasm is greatly vacuolated, and the vacuoles contain different sorts of granules. These granules are the chief differences between types A, B₁, and B₂. Type A contains yellow irregular granules in basal vacuoles around and above the nucleus, and colourless more rounded granules in vacuoles distal to the nucleus, occupying the border of the cell that abuts on the lumen of the tubule. Type B₁ contains colourless granules of the same nature in vacuoles throughout the cytoplasm, and few if any of the yellow granules. Type B₂ has a single vacuole, or very few extremely large vacuoles occupying the greater part of the cell cytoplasm. These contain large, dark brown, rounded bodies. In life the round brown bodies are suspended in a yellow liquid within the vacuole. Type B₂ is distinguished as *Fermentzellen* by the earlier authors; type A corresponds to *Leberzellen*. Cuenot (1892) regards type B₂ as *cellules excrétrices* and type A as *cellules hépatiques*. The results with the histochemical technique described in this paper seem to indicate that this

is the correct interpretation, types B₁ and A being chiefly concerned in secretion.

The localization of β -glucuronidase in the digestive cells. After incubation in accordance with the schedule given, crystals of ferric 8-hydroxyquinoline appear in two principal sites within the cells of the tubule epithelium. The first is in the distal tips of the digestive cells where they abut on the lumen of the tubule, and the second is in the body of the digestive cells, extending down around the basal nuclei, and also out into the tips of the cells. This may be seen in the photomicrographs (fig. 4), which show the heavy nature of the deposit and the complete absence of response in the control section. Careful study of a large number of sections from the experiments shows that there is no constant relationship between the distribution of the crystals and that of the brown bodies of cell type B₂, or that of the yellow irregular granules of cell type A; but the distribution in the sites given above is constant and typical of the tissue. At the highest magnifications it can be seen that the crystals are embedded in the tissue, and often appear to have been formed within the cytoplasmic vacuoles, either around or in close association with the colourless granules. This accords with the gross distribution of the crystals in two principal sites, since it would arise from association with the colourless granules of cell type A and the colourless granules of cell type B₁.

Many crystals appear to be deposited in an irregular manner upon the cut surface of the section, and a large number of these may float off during the various washings. However, crystals which have developed within the tissue consistently show the characteristic distribution. Crystals do not appear to develop in any consistent association with the calcium cells, or with the connective tissue, or the cells of the haemocoelic system. The crystal deposits do not form in all the tubules of a section, which may indicate that different tubules are in different phases of secretory activity.

The histology of the digestive gland of the closely related species *Helix aspersa* is identical with that of *H. pomatia*, and the distribution of the crystal deposits after incubation is also the same.

DISCUSSION

β -glucuronidase appears to be associated with the digestive processes of a number of herbivorous animals. In some it has been suggested that it is the product of intestinal micro-organisms (Marsh and others, 1952). Such organisms have been suggested as responsible for the high activity of the enzyme in the crop fluid of locusts (Robinson and others, 1953). Even higher activities occur in gastropod molluscs, particularly in *H. pomatia*, but the evidence presented in this paper indicates that the enzyme is produced by the tissues

FIG. 4. A, a test section of the digestive gland of *Helix pomatia* after 4 hours' incubation. Unstained. Note the heavy deposits of ferric 8-hydroxyquinoline.

B, a control section. Unstained; the lack of transparency of the tissue is sufficient for photography.

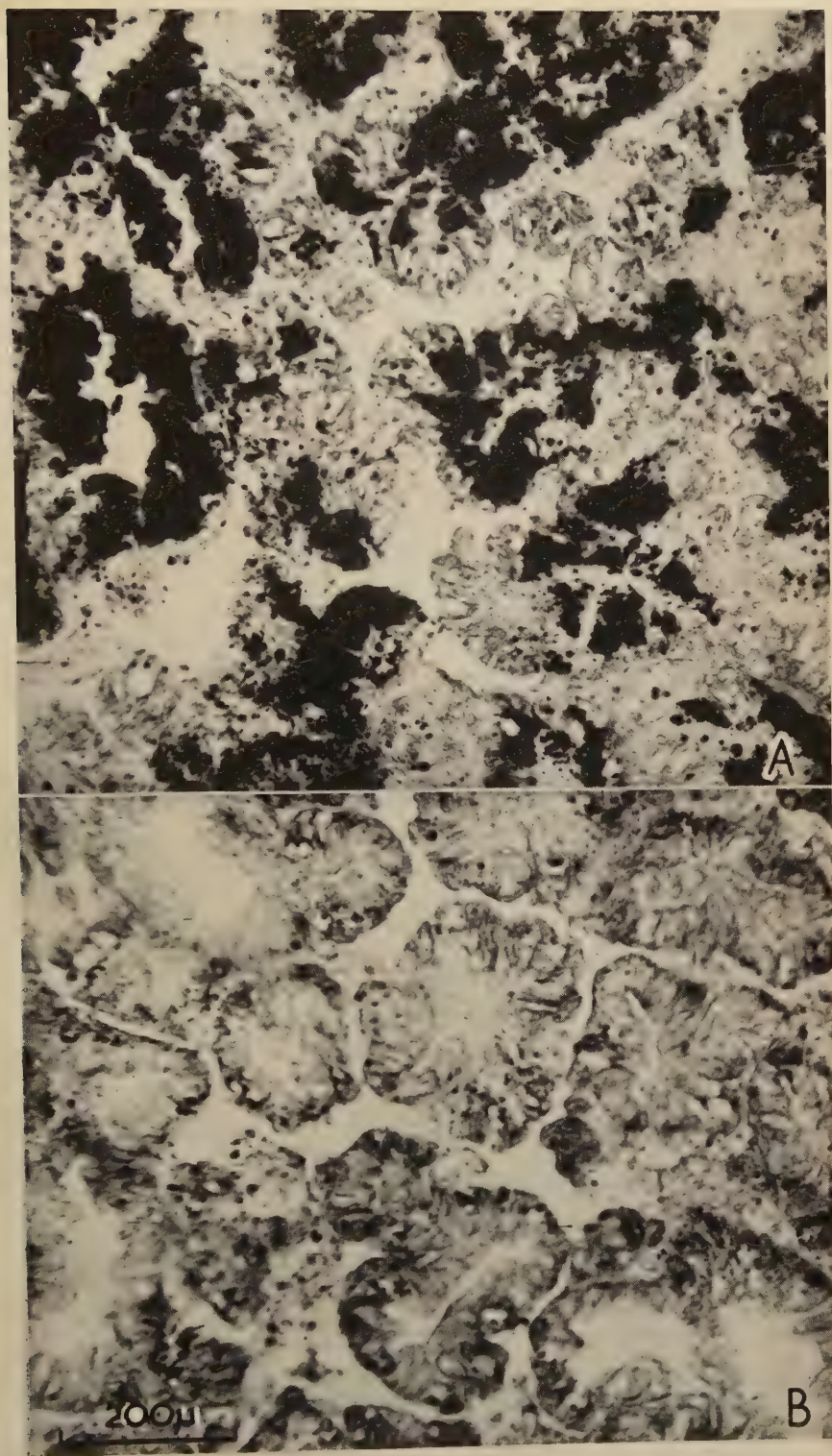


FIG. 4
F. BILLETT and S. M. MCGEE-RUSSELL

of the animal itself, an adaptation probably directly related to the efficient herbivorous digestion by terrestrial snails. It would be interesting to know whether more detailed studies of the locust would demonstrate a similar intrinsic synthesis of the enzyme.

The crop fluid of *H. pomatia* has long been known to contain a variety of enzymes (Biedermann, 1911). Holden and others (1950) review some thirty of the enzymes which have been found in the fluid. It is secreted by two principal secretory organs, the salivary glands and the digestive gland. The greater volume of the fluid appears to be derived from the digestive gland, within which the β -glucuronidase is produced. Recent authors have employed the crop fluid as a useful reagent for the maceration of plant tissue, for there is almost no proteolytic activity in it (Faberge, 1945; Holden, Pirie, and Tracey, 1950). The cellulolytic activity of the fluid is usually attributed to a complex of enzymes termed 'cytase'. At present it is reasonable to suggest that β -glucuronidase is part of this complex. Marsh and others (1952) have suggested hemicellulose as a possible substrate for the enzyme. It is likely therefore that rich sources of β -glucuronidase may be found in wood-boring organisms such as the mollusc *Teredo*, and beetle larvae. Biochemical and histochemical information on such organisms would be valuable.

The modifications in technique given in the paper have the advantages of greater simplicity and increased consistency of results. The principal step of omitting the stage of conversion to Prussian blue appears to give localization as good as, or better than, the original method. This advantage is somewhat offset by the impossibility of making preparations in Canada balsam, but our preparations mounted in Farrant's medium keep well, and avoid the disadvantages of alcoholic dehydration.

Campbell (1949) has suggested that β -glucuronidase is closely associated with the mitochondria of certain cells. This does not appear to be the case with the cells of the snail digestive gland. In view of the large crystal size, it does not appear to us to be possible to demonstrate such an association with the technique as it stands. In any case the conditions of the test, involving the use of acetate buffer, make the survival of mitochondria problematical. Furthermore, cytological detail may be destroyed by autolytic enzymes which have not been completely inactivated by the fixation procedure. The aim of the fixation, in common with other techniques for the localization of enzymes in tissues, is to leave the enzyme as active as possible rather than to preserve cytological detail.

During the course of the experiments with the snail digestive gland it was found that the addition of sodium chloride and potassium sulphate to the substrate mixture produced a more rapid and more intense reaction. This may follow from one of two causes: either the salts may exert a true activating effect on the enzyme, or they may produce a salting out effect, causing a more rapid precipitation of the ferric 8-hydroxyquinoline.

We have applied the technique described in this paper to other tissues. The results obtained confirm the observations made with the snail tissue, but

they demonstrate that variable factors in the tissues, other than the enzyme content, may affect the results of the technique. Different tissues vary in their capacity to adsorb ferric iron. Under certain conditions this factor and the reaction of saccharate with ferric 8-hydroxyquinoline can combine to produce invalid controls (unpublished observations).

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The Cytoplasmic Inclusions of a Mammalian Sympathetic Neurone: A Histochemical Study

By W. G. BRUCE CASSELMAN AND JOHN R. BAKER

(From the Cytological Laboratory, Department of Zoology, University Museum, Oxford)

SUMMARY

1. The neurones studied were those of the anterior mesenteric and coeliac ganglia of immature rabbits.
2. Ectoplasm and endoplasm can be distinguished in these cells.
3. Two kinds of cytoplasmic inclusions occur. These are (a) spherical or spheroid lipid globules or lipochondria, which are confined to the endoplasm; and (b) very minute threads and granules, regarded as mitochondria.
4. A wide variety of histochemical tests was used. The results indicate that the lipochondria consist of galactolipid (cerebrosid) and phospholipid, though the smallest ones may perhaps consist of galactolipid only.
5. The objects regarded as mitochondria are too small for accurate investigation by *in situ* histochemical methods, but they give positive reactions for phospholipid and protein.

OUR object has been to reveal the chemical composition of the cytoplasmic inclusions of the sympathetic nerve-cells of a mammal by the application of reliable histochemical tests. We have not undertaken any enzymological studies. The anterior mesenteric and coeliac ganglia of the rabbit were chosen, partly because they are of convenient size, partly because the former ganglion was used in the earlier studies by one of us (Baker, 1944). We have not detected any difference between the cells of the two ganglia, and the description of our results refers equally to both.

We have not studied the lipofuscin granules that sometimes occur in clumps in the nerve-cells of old animals, the whole of our work having been done on immature specimens, which have no pigment in these ganglia.

MATERIAL AND METHODS

The mean weight of the fourteen rabbits used in the investigation was 0.87 kg. (maximum 1.56, minimum 0.42 kg.). The animals were all killed by breaking the neck suddenly. The ganglia were removed as quickly as possible after death, care being taken not to damage them mechanically. They were at once placed in fixatives. Some (not all) were divided with fine scissors to allow especially rapid access of the fixatives to the nerve-cells.

Sudan black B was used for the localization of lipids. We shall use the expression 'standard Sudan black' to mean that fixation, postchroming, and colouring were done in exact accordance with the instructions given previously by one of us (Baker, 1949). The Nile blue test for the differentiation

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of acidic and neutral lipids (Cain, 1947*a*, 1948) was applied only after fixation in formaldehyde-calcium without postchroming. The acid haematein test for phospholipids (Baker, 1946, 1947*b*; Cain, 1947*b*; Casselman, 1952) and its pyridine extraction control were applied to tissues fixed in formaldehyde-calcium and weak Bouin's fluid respectively. The acid haematein test was also used on ganglia fixed and extracted with cold or hot acetone before being treated with formaldehyde-calcium and the other reagents. Windaus's test for cholesterol (Leulier and Revol, 1926, 1930; Lison, 1953) and Liebermann's for cholesterol and its esters (Schultz, 1924; Schultz and Löhr, 1925) were applied to gelatine sections of ganglia fixed in formaldehyde-calcium.

For the periodic acid / Schiff (PAS) test (McManus, 1946), use was made of gelatine sections that had been fixed in formaldehyde-calcium, Da Fano's, or Regaud's fluid, saturated aqueous mercuric chloride, or Heidenhain's mercuric chloride / saline, or fixed in weak Bouin's fluid and extracted with pyridine (Baker, 1946), or simultaneously fixed and extracted with cold or hot acetone (the treatment with hot acetone being for 18–20 hours in a semi-micro Soxhlet extractor). The PAS test was also done on paraffin sections of ganglia fixed in Carnoy's or Zenker's fluid. Control sections were first acetylated with acetic anhydride in glacial acetic acid (Casselmann, Macrae, Simmons, 1954). The performic acid / Schiff (PFAS) test (Lillie, 1952) was applied to gelatine sections of ganglia fixed in formaldehyde-calcium, or treated with hot or cold acetone, or extracted with pyridine after weak Bouin fixation. Control sections were immersed in saturated aqueous bromine for 3 hours. With both tests, any interference from formaldehyde was prevented by first treating the sections for at least 3 hours with a saturated solution of dimedone in 1 N acetic acid.

Proteins were studied by the Sakaguchi (Baker, 1947*a*) and coupled tetrazonium (Pearse, 1953) tests, which were carried out on gelatine sections of ganglia fixed in Heidenhain's mercuric chloride / formalin or mercuric chloride / saline, in saturated aqueous mercuric chloride, or in Da Fano's or Regaud's fluid, and on paraffin sections of a ganglion fixed in 80 per cent. ethanol.

OBSERVATIONS

The ground cytoplasm

The whole of the ground cytoplasm of the cell is thrown by some of the fixatives into the form of a very fine reticulum, the threads of which are near the limit of microscopical resolution. This appearance is particularly clearly given by fixation in 80 per cent. ethanol and colouring by the coupled tetrazonium test.

The cytoplasm is sometimes seen to contain oval, spindle-shaped, or crescentic spaces, commonly about $2\frac{1}{2}\mu$ long and $\frac{3}{4}\mu$ wide at the widest point. They appear to be empty, for none of our tests has ever given a positive reaction with them. They are most often seen in preparations that have been fixed with solutions containing strong protein precipitants (Carnoy's fluid,

saturated aqueous mercuric chloride, and the mercuric chloride / saline or mercuric chloride / formaldehyde fluids of Heidenhain). They are rendered particularly evident if the cytoplasm is strongly coloured by the coupled

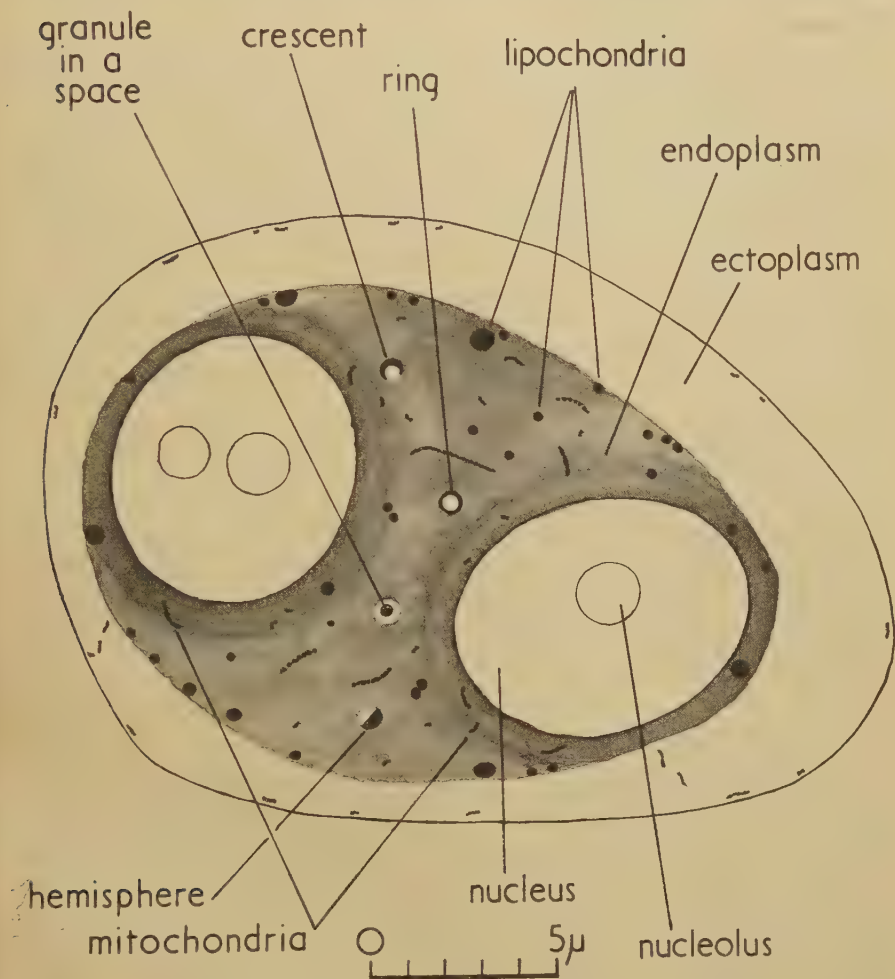


FIG. 1. Diagram of a section through a sympathetic cell (coenocyte) of the immature rabbit. The diagram is based upon what can be seen in a standard Sudan black preparation, but the mitochondria are added from what can be seen in an Altmann-Metzner preparation. The following are regarded as artificially modified lipochondria: granule in a space, crescent, ring, hemisphere.

tetrazonium test. We have not seen them in preparations fixed in formaldehyde-saline, formaldehyde-calcium, or Champy's fluid. It seems probable that they are fixation-artifacts of an unusual kind.

An outer and an inner region can be distinguished in the cytoplasm (fig. 1). This was pointed out in the earlier paper by one of us (Baker, 1944), in which

the inner region was described as the sudanophil or lipoid-containing part. The distinction between the outer and inner parts is particularly easily seen in gelatine sections. We shall call the outer region the ectoplasm and the inner the endoplasm. The width of the ectoplasm appears to vary a good deal in different parts of the same cell.

In our present studies we have found that the ectoplasm generally appears very pale pink in Sudan black / carmalum preparations. It is usually relatively homogeneous in fixed microscopical preparations, and contains few inclusions. It must be mentioned, however, that the ectoplasm tends to be somewhat obscured in many preparations by the lipid globules present in the adjacent capsular or satellite cells. It is sometimes difficult to be sure whether a particle is in the ectoplasm of a neurone or in a capsular cell. This must be borne in mind in reading what follows.

The ectoplasm is negative to the PAS test. The external part of the cytoplasm often reacts somewhat more strongly to the coupled tetrazonium and PFAS tests than the internal part. The two zones are not differentiated by the Sakaguchi reaction.

The endoplasm appears pale blue or blueish grey in Sudan black / carmalum preparations, and is seen to contain many inclusions.

In unstained sections examined by positive phase-contrast microscopy, the cytoplasm appears darker in the immediate vicinity of each of the two nuclei usually contained in the cell (or coenocyte) than elsewhere. These parts of the cytoplasm also tend to react slightly more strongly than the general endoplasm to the coupled tetrazonium test. The modified cytoplasm in the vicinity of the nuclei shades off gradually into the rest.

The cytoplasmic inclusions

Lipocondria. The endoplasm contains colourless lipid globules or lipocondria. They are distributed nearly at random in it, but are more abundant near its outer border than elsewhere. There is no special accumulation of them in the axon hillock. These bodies, most of which are spherical or subspherical, vary considerably in size. Few (if any) of them exceed 1μ in diameter in the immature rabbits we have used. The smallest are below the limit at which reliable measurements can be made; these appear to be 0.2μ or less in diameter. In fixed preparations that have not been treated with any colorant, the lipocondria cannot be distinctly seen by phase-contrast microscopy; they must have nearly the same refractive index as the cytoplasm, after fixation of the cell. They are most easily exhibited by the standard Sudan black technique, which colours them blue-black. Each blue-black globule is sometimes surrounded by a clear space, as though the method of preparation had caused the lipid globule and the surrounding cytoplasm to pull slightly apart from one another. In other cases the lipid seems to have retracted to the edge of a spherical or spheroidal space which presumably represents the original form of the lipocondrion. The coloured material then appears as a hemi-

sphere, crescent, or ring in optical section. The larger lipochondria tend particularly to assume these forms. (See fig. 1.)

After fixation in formaldehyde-calcium or Champy, or fixation in formaldehyde-saline or formaldehyde-calcium with subsequent postchroming, the lipochondria are readily coloured by Sudan black B in gelatine sections. The colorant can be entirely extracted by 70 per cent. ethanol, there being no stable sudanophilia. With Nile blue, the lipochondria cannot be differentiated from the surrounding basiphil cytoplasm. The larger lipochondria give rather a weak, truly positive reaction (AH+, PE-) with the acid haematein test, the reacting material often presenting the forms mentioned in the preceding paragraph. The smaller ones are not stained by acid haematein. When the PAS test is applied to the cell, positively reacting granules are seen which resemble the lipochondria in distribution, number, size, and shape. There seems to be no reason to doubt that they are the same objects as are coloured by Sudan black. The evidence from solubilities, given below, supports this view, and they will be called lipochondria in what follows. The lipochondria cannot be differentiated by the PFAS test nor by the coupled tetrazonium or Sakaguchi tests. They are poorly preserved, if at all, by saturated aqueous mercuric chloride or mercuric chloride / saline, and do not resist paraffin embedding after fixation in Carnoy's or Zenker's fluid or in 80 per cent. ethanol.

The following experiment was done to make certain that none of the lipochondria seen with Sudan black B was merely due to precipitation of the reagent. A standard Sudan black preparation was mounted in glycerine. One particular cell was selected and the distribution of the twenty lipochondria seen in one plane of focus was recorded in a careful drawing. The lipid colorant was then removed with 70 per cent. ethanol. The section was again mounted in glycerine and the complete removal of the Sudan black from the cell confirmed. It was again coloured with Sudan black B as before. Re-examination of the same cell in the same plane of focus showed the same twenty lipochondria that had been recorded in the drawing prepared earlier, each characterized by its position and size, and in some cases by its shape.

The lipochondria respond negatively to the tests for cholesterol and for its esters.

When a freshly removed ganglion is placed in acetone at room temperature, left in this overnight, and then subjected to the acid haematein test, the positively reacting material appears in a diffuse form, not as separate lipochondria and mitochondria. The phospholipid content of the cell appears to be pushed inwards by the invading acetone, but it cannot pass through the cell-membranes and therefore tends to accumulate on the side of each cell nearest the centre of the piece of tissue. There is a gradient in the intensity of the blue-black reaction, which falls off to nothing on the side of each cell that is nearest to the outside of the piece of tissue. We have made no attempt at quantitative studies, but it appears that more phospholipid is revealed after the previous action of acetone than is seen in all the lipochondria and mitochondria after the application of the ordinary acid haematein test without previous treatment

with acetone. This suggests that there is a considerable amount of histochemically unreactive phospholipid in the cell, and that acetone sets it free.

When a ganglion that has been fixed as above in cold acetone is subsequently fixed and coloured according to the standard Sudan black technique, the diffuse lipid is coloured pale grey. The lipochondria are coloured by the Sudan black in some of the cells, but they often appear as crescents, rings, or very small dots, as though part of their material had been extracted. They give a positive PAS reaction, scarcely diminished in intensity from that seen in sections of ganglia not treated with acetone.

The lipochondria are not demonstrable by any method in sections of ganglia treated overnight with hot acetone in a Soxhlet extractor. The displaced phospholipid resists such treatment and retains its histochemical properties. Neither lipochondria nor displaced lipids are present after pyridine extraction.

The lipochondria are not coloured by Metzner's method after Altmann fixation.

Mitochondria. It is usually difficult to make clear preparations of the mitochondria of the nerve-cells of mammals, and the cells that are the subject of the present paper constitute no exception in this respect. If ganglia are fixed for 24 hours in Altmann's fluid, sectioned at 2 or 3 μ in paraffin, and stained by Metzner's method (Metzner and Krause, 1928), with very careful differentiation, the cytoplasm is seen to be yellow, with numerous very small pink or red objects in it. These objects appear to be extremely thin threads and tiny granules, but the threads are so thin and the granules so minute that the resolution obtainable by the light-microscope does not permit any exact description of their form or width, though the threads sometimes appear to be made up of rows of granules. The longest threads are as much as 3 μ in length. These bodies, which are not seen in routine preparations of any kind, are presumably mitochondria. The threads resemble the mitochondria seen by Thomas (1948) in the living neurone of the anterior mesenteric ganglion of the mouse.

It would appear that the mitochondria occur mainly in the endoplasm, but that there are also some in the ectoplasm, especially in its outermost part. Ectoplasm and endoplasm are not clearly differentiated, however, in Altmann-Metzner preparations, and the ectoplasm seems to be considerably shrunken.

Only two of the many histochemical tests that we have applied show any objects that could be regarded as mitochondria. These are the acid haematein and coupled tetrazonium techniques. In acid haematein preparations the cytoplasm appears yellowish with extremely fine blue or blue-grey markings. When these markings are examined critically with the highest useful powers of the microscope, they can just be resolved into threads and granules, which appear to have the same distribution as the objects seen in Metzner preparations. Exceedingly thin, positively reacting threads are also seen in the cytoplasm of cells that have been fixed in mercuric chloride solution, sectioned in gelatine, and subjected to the coupled tetrazonium test. The positive reactions

with acid haematein and coupled tetrazonium are consistent with the identification of these bodies as mitochondria.

Other cytoplasmic inclusions. A supposedly canalicular object has been reported by Lacy (1954a) in the exocrine cell of the mammalian pancreas, and it has been thought that this may represent a cytoplasmic inclusion of quite general occurrence in the cells of vertebrates. We have used Lacy's method: that is to say, we have fixed and postchromed ganglia as for the standard Sudan black method, cut very thin gelatine sections, and coloured them with Sudan black B. We have prolonged the period of colouring to 20 minutes (instead of the usual $2\frac{1}{2}$), because this is said to make it easier to see the canals in the pancreas (Lacy, 1954b). A careful study of these preparations has not revealed any canalicular object resembling what Lacy reports in the pancreatic cell.

In all our histochemical preparations, very variously fixed and coloured, we have looked attentively for any cytoplasmic inclusion having the form of a network, but have not found anything of this kind.

DISCUSSION

The lipochondria described in this paper are clearly the same as those already described by one of us in the anterior mesenteric ganglion of the rabbit (Baker, 1944, p. 30). They correspond to the 'spheroids' studied by Thomas (1948) in the same ganglion of the mouse. Perhaps they correspond also to the bodies figured by Ciaccio in spinal ganglion cells of the dog (1910, his plate XIX, figs. 28 and 29), though these might have been lipofuscin pigment.

From the histochemical observations, we conclude that the lipochondria have two main components. The more plentiful one is sudanophil and insoluble in cold acetone but soluble in hot acetone or hot pyridine, and gives a positive reaction with the PAS test which is blocked by acetylation but a negative one with the acid haematein test. On the basis of these properties, it can be classified as a galactolipid (cerebroside). The other main component, which cannot be demonstrated with certainty in the smallest lipochondria, is sudanophil, insoluble in hot or cold acetone but soluble in hot pyridine, and gives a negative reaction with the PAS test but a truly positive one with the acid haematein test. This can be classified as phospholipid. Thus the composition of at least the larger lipochondria in the rabbit sympathetic neurone studied by us is qualitatively similar to that of the lipochondria in the locust neurone (Shafiq and Casselman, 1954). Although quantitative interpretation of histochemical tests should be undertaken with great caution, it appears that in the lipochondria of the rabbit neurones, galactolipid predominates (or actually may occur alone in the smaller globules), whereas in the locust neurones the reverse is true. It is likely that the 'diffuse' glycolipid described by Dixon and Herbertson (1950b, 1951) as occurring in various cells of the brain of the rabbit, corresponds to the cerebroside component of the lipochondria we have studied. They themselves note (1950b) that it can sometimes

be recognized as occurring in the form of 'extremely fine cytoplasmic granules'.

There is no reason to believe that the lipochondria which we have studied are identical with the sudanophil and PAS-positive granules described by Dixon and Herbertson (1950, *a* and *b*) in human and rabbit neurones. As these authors point out, their granules vary in colour from pale yellow to dark brown, owing to the presence of lipofuscin pigment in them. Further, their granules are very resistant to lipid-solvents, and indeed were studied in paraffin sections, generally after simple fixation in formaldehyde-saline, without postchroming. It must be mentioned that lipofuscin granules have a strong tendency to be aggregated in a particular part of the cell, and indeed this is shown in most of the figures of Dixon and Herbertson (1950*b*), while lipochondria are distributed throughout the endoplasm. It is possible, and even probable, that lipofuscin develops in certain lipochondria as the animal ages by autoxidation. It was for this reason that we used immature rabbits exclusively.

We thank Prof. A. C. Hardy, F.R.S., for his continued support and encouragement of our cytological studies. As usual, Miss B. M. Jordan has given a lot of practical help. This investigation was carried out during the tenure of a Merck Postdoctoral Fellowship in the Natural Sciences of the National Research Council of Canada by one of us (W. G. B. C.), while on leave of absence from the Banting and Best Department of Medical Research, University of Toronto.

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The Cytochemistry of the Amoebocytes and Intestinal Epithelium of *Venus mercenaria* (Lamellibranchiata), with Remarks on a Pigment resembling Ceroid

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With two plates (figs. 1 and 2)

SUMMARY

The properties of the amoebocytes and intestinal epithelium of *Venus mercenaria* were studied by a variety of cytochemical procedures designed to demonstrate proteins, enzymes, carbohydrates, and lipids.

The cytoplasm of the amoebocytes contains specific granules which are constantly present and which are interpreted as being atypical mitochondria. Identification of their mitochondrial nature rests on their staining with Janus green B, their positive reaction for phospholipid by Baker's test, and the presence of dehydrogenase activity. Unlike typical mitochondria, the specific granules are eosinophil. Protein-bound carbonyl groups and disulphide and sulphhydryl groups are present in both the specific granules and the cytoplasm. The sulphhydryl groups may in part be associated with the presence of dehydrogenase, lipase, and serum cholinesterase. Amoebocytes also contain glycogen and a material that is resistant to diastase and positive to the periodic acid / Schiff test; this material may be a neutral polysaccharide, unsaturated lipid, or mucoprotein.

Cytoplasmic structures which are inconstantly present in amoebocytes include sudanophil droplets, neutral red vacuoles, metachromatic granules, and granules of an excretory pigment resembling ceroid. The sudanophil droplets may be stored neutral fat or lipid associated with the Golgi apparatus. The neutral red vacuoles are not preformed inclusions, but form as the dye accumulates within the cells. Metachromatic granules, which are confined solely to the intestinal amoebocytes, consist of phagocytosed intestinal mucus liberated from goblet cells.

The histochemical reactions of the columnar intestinal epithelium suggest that these cells may be active in the digestion and absorption of nutrients, since eosinophil granules, lipid droplets, alkaline phosphatase, lipase, and serum cholinesterase are present in them.

Masses of a ceroid-like excretory pigment and goblet cells containing mucus are present between the columnar intestinal epithelial cells. The pigment contains phospholipid and apparently arises as an oxidized end-product of lipid metabolism.

AMOEBOCYTES are granulocytes which are found in large numbers in the blood, connective tissues, gills, cardiac muscle, and intestinal mucosa of invertebrates. They have been the subject of numerous morphological and chemical studies and many speculations have been advanced concerning their nature and functions (Metschnikoff, 1884; Haughton, 1934; Takatsuki, 1934; Ohuye, 1938; Yonge, 1923, 1926a, 1926b, 1946).

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The present study concerns the cytology and cytochemistry of the amoebocytes and columnar intestinal epithelium of the quahog, *Venus mercenaria*. A ceroid-like excretory pigment found in these cells as well as in other tissues of *Venus*, was also investigated. Interest in these topics arose from a previous investigation of esterolytic enzymes in *V. mercenaria* (Zacks and Welsh, 1953).

The individual amoebocyte is a nucleated cell 9–12 μ in diameter, which, in recently drawn blood, rounds up and appears as a sphere with small projecting spikes 1–2 μ long. After remaining in contact with the surface of a glass slide for several minutes, or in tissue-spreads, the amoebocytes extend pseudopods; by means of these they are capable of slow motion. Within their cytoplasm, small hyaline granules as well as yellow, refractile granules of excretory pigment are seen. The numerous, even-sized, hyaline granules are constantly present in *Venus* amoebocytes and therefore will be termed specific granules to distinguish them from granules of excretory pigment and other cytoplasmic inclusions which are inconstant constituents.

The chemical nature of the cytoplasmic granules of these amoebocytes has interested a few investigators, but no one appears before to have studied the granules by cytochemical methods. By these procedures it was hoped that information might be obtained concerning the chemical nature of the granules and their relations to the enzymatic activities of the cells. The chemical cytology of the columnar intestinal epithelium was investigated with particular reference to its role in digestion and absorption of nutrients. The nature of the excretory pigment which is present in the amoebocytes, intestinal epithelium, and other tissues of *Venus* was similarly investigated by histochemical means. This pigment, which occurs in the form of granules in the amoebocytes and amorphous clumps in the intestine, has been described as a degradation product of chlorophyll (MacMunn, 1900) and as echinochrome (Durham, 1891). Since the chemical nature of this pigment is poorly understood, it was hoped that histochemical tests might contribute to its characterization.

MATERIALS AND METHODS

Fresh specimens of *V. mercenaria* were purchased from a fish dealer. Whole hearts were excised and placed either in sea-water for supravital staining or in various fixatives. Hearts prepared in this way were convenient objects for the study of both amoebocytes and intestinal epithelium, since the heart is wrapped around a segment of intestine.

Supravital methods. For supravital staining, whole hearts were placed in sea-water containing *Janus green B* at 1:10,000 or *neutral red* at 1:10,000 and stained for 2 hours at 29° C. A fragment of atrial wall was then spread under a coverslip on a glass slide for examination. This preparation was chosen because the thin network of atrial muscle fibres enmeshed and supported the amoebocytes, thus allowing a favourable opportunity for observing pseudopod formation and amoeboid motion.

Methods for the staining of proteins. Basophil structures were studied in Zenker-fixed material. Deparaffinized sections were stained in methylene blue solutions buffered at pH 2.3, 4.3, 6.4, and 7.4 according to the method of Singer and Morrison (1948). Similar sections were also stained by Mallory's eosin and methylene blue. The Feulgen procedure, followed by counter-staining with light green, was applied to deparaffinized sections to detect desoxypentose-nucleic acid (DNA). Protein-bound sulphydryl and disulphide groups were sought in deparaffinized sections of hearts fixed in 80 per cent. alcohol containing 1 per cent. trichloroacetic acid (Barnett and Seligman, 1952). Sections were stained with and without prior reduction in $(\text{NH}_4)_2\text{S}$, to allow identification of both sulphydryl and disulphide groups.

Methods for the detection of enzymes. Gomori's acid and alkaline phosphatase methods (1939, 1941), as modified by Dempsey and Deane (1946), were applied to alcohol- and acetone-fixed deparaffinized sections as well as to fresh frozen sections. Sections were incubated for 30 minutes, 1, 3, 6, and 24 hours in solutions of glycerophosphate buffered to pH 4.5 and 9.5.

Dehydrogenase activity was detected in fresh hearts after incubation for 20 minutes in blue tetrazolium chloride (pH 7.4), a method modified from that of Rutenburg, Gofstein, and Seligman (1950).

Substances capable of reducing ferricyanide to ferrocyanide were studied by applying the ferric-ferricyanide reaction of Lillie and Burtner (1953) to fresh and formalin-fixed sections.

Methods for the detection of carbohydrates and mucoproteins. Glycogen and polysaccharides were stained by the periodic acid / Schiff (PAS) method of McManus (1946) and Hotchkiss (1948), after fixation of hearts in Rossman's fluid. Control sections were treated with diastase before application of this procedure. Metachromasia was investigated by staining with 0.5 per cent. toluidine blue after 4 per cent. lead acetate fixation (Holmgren, 1940; Jorpes, Holmgren, and Wilander, 1937).

Methods for the detection of lipids. The distribution of lipids was studied after staining frozen sections of fresh and formalin-fixed hearts with Sudan black B. Phospholipids were identified in frozen sections of hearts fixed in formaldehyde-calcium and in weak Bouin's fluid (Baker, 1946). Carbonyl groups were sought in frozen sections of material fixed in buffered 10 per cent. formalin and stained by the method of Ashbel and Seligman (1949). Control sections were extracted with acetone to remove lipid-containing carbonyl groups. Cholesterol and cholesteryl esters were investigated in frozen sections by the Schultz procedure and acid-fast substances were identified in material fixed in formalin, alcohol, and Zenker's fluid, by the carbol-fuchsin procedure of Lillie (1948).

Methods for the detection of birefringence and fluorescence. Birefringence and fluorescence were studied in fresh-frozen and formalin-fixed sections.

Method for the detection of iron salts. To detect the presence of iron salts material fixed in 80 per cent. alcohol and in Zenker's fluid was embedded in paraffin, sectioned, and treated with potassium ferrocyanide (Lillie, 1948).

OBSERVATIONS

Supravital staining. Spreads of atrial muscle stained in 1:10,000 Janus green B exhibited amoebocytes which were uniformly filled with deep blue-green granules 1–2 μ in diameter. The harmlessness of this dye was shown by the slow extension of pseudopods from the cells. Withdrawal of pseudopods was never observed in supravitaly-stained or fresh, unstained preparations. After 3–5 minutes, the specific granules appeared red-violet in colour, and eventually assumed the red colour of the reduction-product of Janus green B, diethyl safranin. Recolouring of the granules did not occur when the atrial fragments were re-exposed to atmospheric oxygen.

The cut ends of the intestine appeared deep blue-green after staining in Janus green B and this staining was almost entirely confined to the intestinal epithelium. A faint blue-green colour was observed in the ventricular muscle, but the intestinal muscle was nearly devoid of staining.

When neutral red (1:10,000) was applied supravitaly for 2 hours, the amoebocytes were filled with deep brick-red inclusions of various sizes (fig. 1, A). The majority of the neutral red inclusions were larger and less uniform in size than the granules stained by Janus green B. The brick-red colour of the neutral red inclusions suggested that their pH was close to neutrality.

Excretory pigment was unstained by Janus green B and neutral red.

Basiphilia. In sections stained in methylene blue at pH 7.4 and 6.4, cardiac and intestinal muscle fibres, goblet cell mucus, and excretory pigment were stained deep blue, whereas the cytoplasm of the amoebocytes was moderately stained blue. After staining with methylene blue at pH 4.3, the intestinal muscle fibres were coloured grey-blue, amoebocyte cytoplasm was stained light blue, and masses of excretory pigment appeared blue-green. Goblet cell mucus was metachromatically stained deep violet at pH 3, but excretory pigment and cardiac and intestinal muscle fibres and amoebocyte granules were unstained. However, the cell plasma of the amoebocytes was faintly stained blue. At pH 2, nothing was stained.

FIG. 1 (plate). A, atrial muscle spread, illustrating the appearance of amoebocytes supravitaly stained by neutral red. Large and small irregular vacuoles are present in each cell.

B, transverse section through the intestine of *Venus mercenaria*, stained by the Feulgen reaction and counterstained by light green. The typical ciliated columnar epithelium, as well as numerous amoebocytes and accumulations of excretory substance, are illustrated. Numerous amoebocytes are scattered between the intestinal muscle fibres.

C, an amoebocyte stained by the Feulgen procedure and counterstained with light green. The cytoplasm is packed with green-stained specific granules.

D, a goblet cell after application of the Feulgen procedure. The goblet cell contains red-stained mucus and the nuclear chromatin is coloured red-violet.

E, higher magnification of the field shown in B. Masses of excretory pigment composed of globules of yellow or brown material embedded in a green-stained matrix are illustrated.

F, transverse, alcohol-fixed section of *Venus* intestine after 3 hours' incubation in glycerophosphate at pH 9.5. Alkaline phosphatase activity is located in the distal portion of the epithelium. Excretory pigment is unreactive yet some clumps of this material appear dark in the photograph due to the intrinsic colour of the pigment.

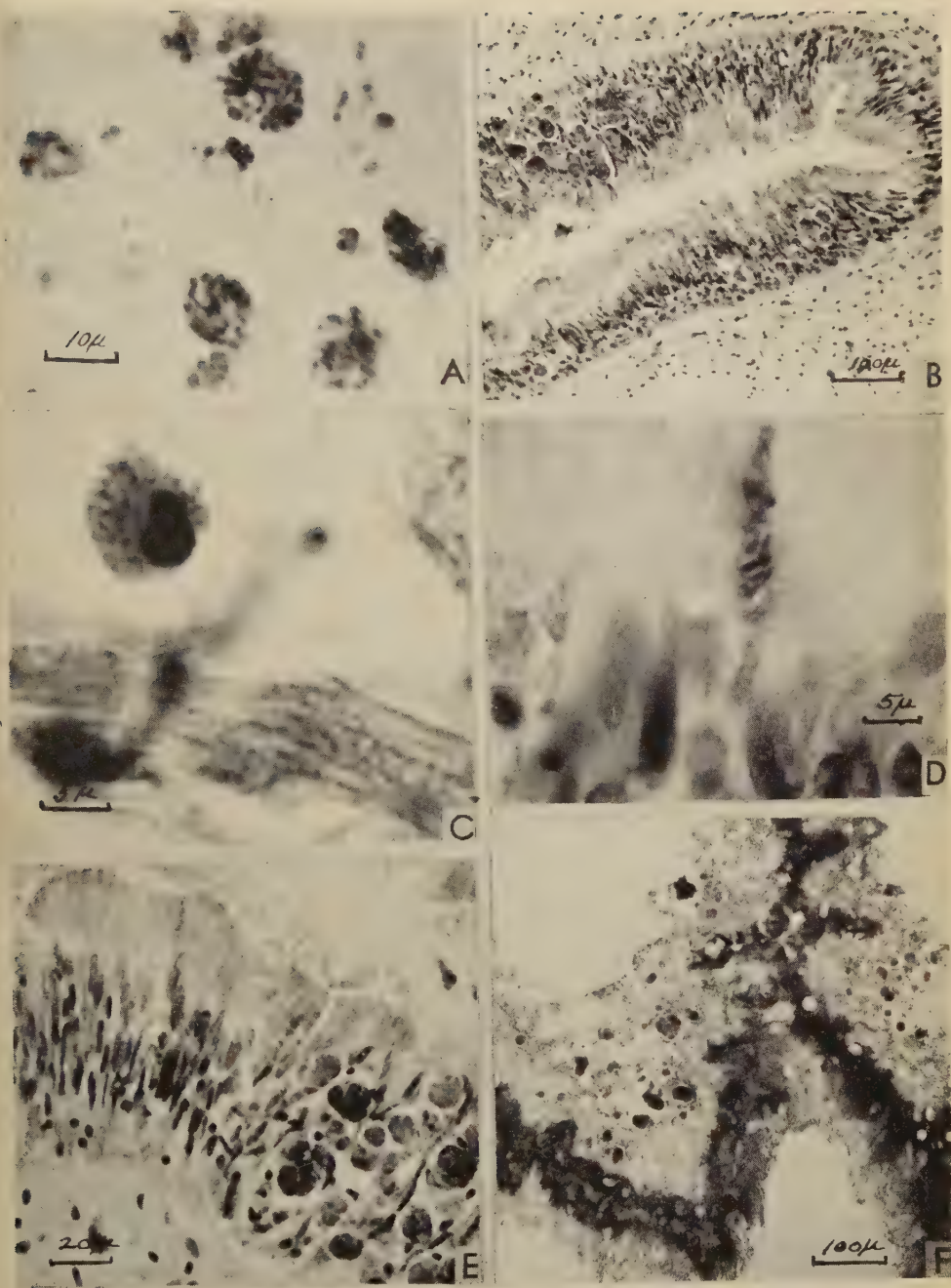


FIG. 1
S. ZACKS

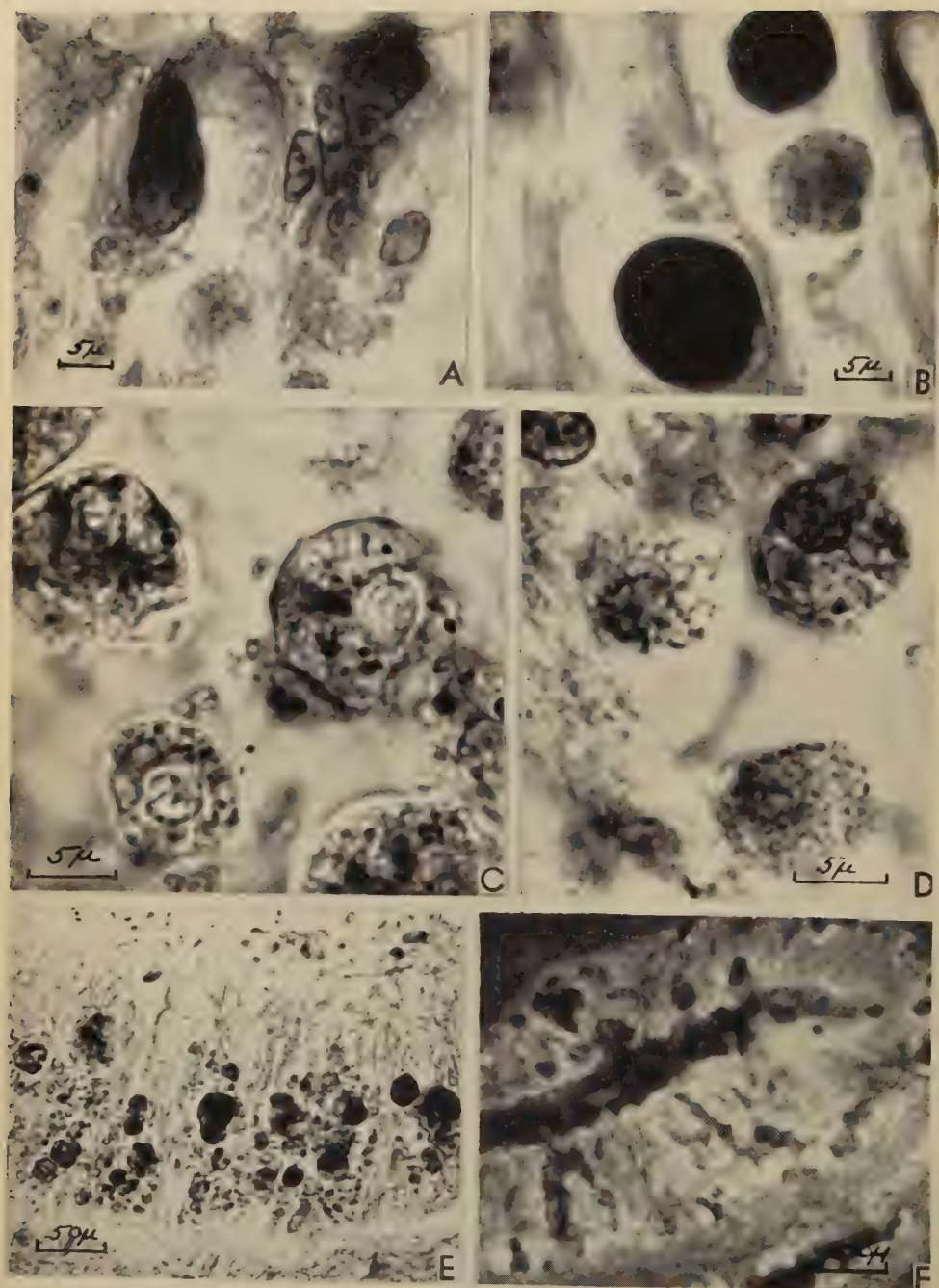


FIG. 2
S. ZACKS

Eosin and methylene blue. After staining with eosin and methylene blue (pH 5.3), the specific granules of the amoebocytes were stained red. Occasional amoebocytes contained a few blue granules of various size. The oval, peripherally placed nucleus contained strongly basiphil clumps of chromatin.

The intestinal epithelium was composed of tall, ciliated, pseudostratified columnar cells which rested on a thick basement membrane. The proximal and middle portions of each epithelial cell were basiphil and numerous dust-like eosinophil granules were present in the distal zone. The intestinal surface of the epithelial cells was equipped with cilia which were $7-10\mu$ in length. The nucleus was located in the middle or proximal part of the cells. Amoebocytes containing specific granules were gathered in great numbers on the basement membrane in spaces between the epithelial cells. Numerous amoebocytes were also present between the smooth muscle fibres of the intestinal musculature. Occasional goblet cells, containing deeply basiphil mucus, were present between the columnar epithelial cells.

Masses of various sizes ($7-30\mu$) of amorphous, strongly basiphil excretory pigment were also present in the spaces between the epithelial cells. Small granules of the same material were scattered between the cardiac muscle fibres.

Desoxyribonucleoprotein. The Feulgen procedure stained the nuclei of the muscle fibres, epithelial cells (fig. 1, B) and amoebocytes, but the specific granules of the amoebocytes were not coloured (fig. 1, C). The cytoplasm of the goblet cells was stained deep red in contrast with the violet colour of the nuclei (fig. 1, D). The cytoplasm of the amoebocytes and portions of the excretory pigment masses were stained by the acid dye, light green, used as counterstain. The excretory pigment masses in the intestinal epithelium were composed of unstained yellow or orange globules embedded in a green-stained matrix (fig. 1, E).

Protein-bound sulphhydryl and disulphide groups. After $(\text{NH}_4)_2\text{S}$ reduction, the intestinal epithelium, intestinal muscle cells, and cardiac muscle reacted positively. The amoebocytes also contained sulphhydryl groups, as indicated by a positive reaction of their cytoplasm. It could not be ascertained whether the reactive material was localized in both the specific granules and cell plasma of the amoebocytes, or only in the cell plasma.

Amoebocytes, intestinal epithelium, and ventricular muscle also reacted

FIG. 2 (plate). A, a goblet cell after 3 hours' incubation in glycerophosphate at pH 9.5. The goblet cell mucus shows alkaline phosphatase activity.

B, transverse section of the intestinal epithelium illustrating the appearance of an amoebocyte and two masses of excretory pigment stained by the PAS-procedure.

C, amoebocytes stained by Sudan black B, showing lipid droplets.

D, amoebocytes stained by the Baker procedure showing numerous phospholipid granules in the cytoplasm.

E, transverse section of *Venus* intestine after staining for phospholipid. The masses of excretory pigment contain phospholipid granules embedded in a matrix of unstained material.

F, ultra-violet light photograph illustrating the intense fluorescence of the excretory pigment and the less intense fluorescence of the intestinal epithelial cells.

positively when reduction with $(\text{NH}_4)_2\text{S}$ had been omitted, thus indicating the presence of free-SH groups as well as S-S groups in these cells. By both procedures, the excretory pigment was unreactive.

Alkaline and acid phosphatases. Alkaline phosphatase activity was more intense in fresh-frozen sections than in alcohol-fixed sections. After 30 minutes' incubation, the distal ends of the columnar epithelial cells were stained brown (fig. 1, F) but the amoebocytes and excretory pigment were unreactive. After 6 hours of incubation the intestinal epithelium was intensely stained, but only an occasional amoebocyte could be found which contained one or two brown granules. In alcohol-fixed sections, the goblet cells were stained brown after 3 hours of incubation (fig. 2, A).

Acid phosphatase activity was not demonstrable in any of the tissues investigated.

Dehydrogenase. After 20 minutes' incubation in blue tetrazolium chloride, granules of blue formazan pigment were present in the amoebocytes and the cardiac and intestinal muscle fibres.

Ferric-ferricyanide reaction. After 10 minutes in this reagent, the cardiac muscle and intestinal epithelium were tinged light green, the amoebocytes were colourless, and the masses of excretory pigment were coloured deep blue-green.

Periodic acid / Schiff reaction. The PAS procedure produced intense red staining of the cardiac and intestinal muscle fibres, goblet cells, and excretory pigment. The amoebocytes were filled with many red granules of various sizes. Fig. 2, B illustrates the appearance of an amoebocyte and masses of excretory pigment stained by this procedure. After the exposure to the action of diastase to remove glycogen, staining of both cardiac and intestinal muscle fibres and amoebocytes was considerably reduced. However, goblet cells and excretory pigment were deeply stained despite exposure to diastase.

Staining with toluidine blue (metachromasia). Muscle fibres, the specific granules of the amoebocytes, and the excretory pigment were stained faintly blue.

Metachromatic granules and amorphous clumps were abundantly present in the intestinal lumen and epithelium as well as in amoebocytes located between the epithelium. However, large numbers of amoebocytes located in the intestinal and cardiac musculature were devoid of metachromatic granules.

Lipids. After the staining of atrial spreads in Sudan black B, each amoebocyte contained up to a dozen black droplets of variable size. The larger droplets were occasionally U shaped and associated with cytoplasmic vacuoles. A tendency toward perinuclear localization occurred (fig. 2, c). After 15 minutes' extraction in acetone or hot alcohol (60°C .), the sudanophil droplets could no longer be demonstrated.

The amorphous masses of excretory pigment were stained green-black or grey-green by Sudan black B and small black sudanophil droplets were present in the apices of the intestinal epithelial cells.

Phospholipids. Each amoebocyte contained 2-50 black granules of uniform

size (fig. 2, D) after staining by Baker's method for phospholipids. In many amoebocytes, these granules were not as numerous as the specific granules which were stained by supravital Janus green B or eosin. After pyridine extraction, the nuclei of the amoebocytes and muscle cells were stained, but the granules were unstained. These results indicate that the amoebocyte granules contain phospholipid. It should be emphasized, however, that in many amoebocytes, the phospholipid granules represented but a small fraction of the full complement of granules stained by Janus green B or eosin.

Black-stained granules and clumps were embedded in the yellow masses of excretory pigment present in the intestinal columnar epithelium which was otherwise unstained (fig. 2, E).

Carbonyl groups. The distal halves of the columnar intestinal epithelial cells reacted strongly for carbonyl groups, but the basal halves stained only faintly. The musculatures of both heart and intestine were unreactive. The specific granules as well as the cell plasma of the amoebocytes were moderately stained. In control preparations extracted with acetone, the outer portions of the epithelial cells remained reactive, but the masses of excretory pigment were stained red-orange, a result attributable to non-specific solution of the blue azo dye in the lipid component of the excretory pigment (Nachlas and Seligman, 1949). Carbonyl-staining was reduced in amoebocytes after acetone extraction.

The Schultz test for cholesterol and cholesterol esters was negative in all of the cell elements studied.

Acid-fast substances. After several hours' extraction in dilute hydrochloric acid, red masses of excretory substance and occasional small granules in amoebocytes were present.

Birefringence and fluorescence. Neither epithelium, excretory pigment, nor amoebocytes were birefringent when examined under the polarization microscope.

In ultra-violet light, the middle and basal portions of the columnar intestinal epithelial cells showed light blue fluorescence, whereas the apical, granular portion of the cells exhibited red-violet fluorescence. The large, amorphous masses of excretory pigment situated on the basement membrane and between the epithelial cells (fig. 2, F), as well as occasional small granules in the amoebocytes, showed intense yellow fluorescence. Similar fluorescent granules were scattered between the intestinal muscle fibres. Much of this material seemed to be free, but a smaller fraction was located within amoebocytes.

Ferrocyanide reaction (iron salts). Sections stained by this means were completely negative, no blue or green deposits of Prussian blue being observed.

DISCUSSION

The nature and properties of the various structures in the cytoplasm of amoebocytes

A variety of cytoplasmic inclusions which exhibit distinctive histochemical properties and enzymatic reactions were present in the amoebocytes of

V. mercenaria. These included specific granules (constantly present), neutral red vacuoles, sudanophil droplets, pigment granules, and metachromatic and PAS-positive materials, all of which were inconstantly present. Besides the reactions shown by these various inclusions the cell plasma itself manifested certain staining properties.

Cytochemical reactions of specific granules in amoebocytes

Supravital staining with Janus green B showed great numbers of even-sized *specific granules* which reduced the dye to diethyl safranin, thus indicating the presence of hydrogen-donor enzymes. That this reaction was not reversible was seen in the failure of the specific granules to become coloured again when exposed to atmospheric oxygen. The fact that these granules stained with Janus green B and were capable of reducing this dye when oxygen was excluded, indicated that they are of mitochondrial nature. Furthermore, dehydrogenase activity associated with the specific granules was indicated by the oxidation of blue tetrazolium chloride. The mitochondrial nature of the specific granules was also suggested by the fact that many of the granules reacted positively with Baker's test for phospholipid. However, the specific granules were eosinophil, a staining reaction which does not occur in typical mitochondria. The foregoing observations suggested that amoebocyte specific granules represent an atypical variety of mitochondria.

Diastase-labile as well as diastase-resistant *PAS-positive granules* were present in the amoebocytes. The substances responsible for this reaction are thought to be compounds containing 1, 2 glycol linkages which are oxidized by periodic acid to form aldehyde groups which then react with the Schiff reagent (Hotchkiss, 1948). According to Leblond (1950), only substances insoluble in water and fat substances can be considered to persist after exposure of the tissues to the reagents employed in fixation and paraffin embedding. These substances include glycogen, which can be removed by pretreatment of the sections with diastase, and mucopolysaccharides and mucoproteins which are not removed by diastase. Lillie (1950), Wolman (1950), and Pearse (1953) have shown that certain lipids also yield a positive PAS-reaction which is retained after exposure to the action of diastase. Unsaturated lipids or other substances containing hydroxyl and amino groups on two adjacent carbon atoms might be expected to yield a positive PAS-test.

Since much of the material which was stained by the PAS procedure was removed by the action of diastase, it appears that these cells contain glycogen. However, amoebocytes also contained granules of diastase-resistant material.

The nature of the diastase-resistant material in amoebocytes is not clear. This material may be neutral polysaccharide, since evidence of strongly acidic groups of acid mucopolysaccharides is lacking, or mucoprotein, phospholipid, or other unsaturated lipid. The presence of neutral fat and phospholipid as well as PAS-positive excretory pigment has been demonstrated in *Venus* amoebocytes. Furthermore, since serum cholinesterase is present in amoebocytes (Zacks and Welsh, 1953), it is of interest that cholinesterase is

thought to be a mucoprotein and that practically all sites possessing cholinesterase activity are PAS-positive (Gomori, 1951). Thus the diastase-resistant material may be unsaturated lipid or mucoprotein.

The presence of *ketonic carbonyl groups of proteins* or proteo-lipids was indicated rather than ketonic groups associated with lipids (Seligman and Ashbel, 1951) since the amoebocytes were still reactive after acetone extraction.

Sulphydryl and *disulphide* groups were present in both the specific granules and cell plasma of amoebocytes. These groups are important in binding proteins and prosthetic groups (Barron, 1951), cell division and growth (Brachet, 1950), cell permeability (Lefevre, 1948), and in enzymatic activity (Barron, 1951). Several sulphydryl enzymes have been demonstrated in amoebocytes. The presence of lipase in the amoebocytes of several molluscs has been shown by Yonge (1926*b*) and Takatsuki (1934), and recently by histochemical procedures by Zacks and Welsh (1953). Serum cholinesterase is also present in the amoebocytes of *Venus* as indicated by carbonaphthoxycholine iodide hydrolysis (Zacks and Welsh, 1953). In addition, Takatsuki (1934) has demonstrated enzymes capable of attacking starch, glycogen, maltose, lactose, sucrose, salicine, and gelatine in extracts of *Ostrea* amoebocytes.

Among the numerous enzymes requiring SH-groups for their activity are cholinesterase, lipase, esterase, β -amylase, and carboxypeptidase (Barron, 1951). Of this group, cholinesterase, lipase, and an unidentified dehydrogenase have been detected in *Venus* amoebocytes, and amylase, protease, and lipase have been demonstrated in the amoebocytes of other species (Yonge, 1926*b*; Takatsuki, 1934). Since several sulphydryl enzymes are present in amoebocytes, it seems that the sulphydryl groups demonstrable in these cells may be partially attributed to these enzymes.

Metachromasia in amoebocytes. The absence of metachromatic granules in amoebocytes outside the intestine suggests that intestinal amoebocytes which contain metachromatic granules had phagocytosed some of the goblet cell mucus. Metachromatic staining is regarded as evidence for the presence of acid mucopolysaccharides (Holmgren and Wilander, 1937; Wislocki, Bunting, and Dempsey, 1947), an important constituent of mucus from several sources. Mucus is used by lamellibranchs to trap food particles to aid their ingestion by amoebocytes and digestive diverticula (Yonge, 1926*b*).

Sudanophil droplets in amoebocytes. Sudanophil droplets of various size were present in nearly all the amoebocytes. In every case these droplets could be distinguished from the specific granules by their relatively small number and by their size and position within the cell. Since the sudanophil droplets were extractable by cold acetone and hot alcohol, they appeared to consist of neutral fat.

The presence of lipids in lamellibranch amoebocytes has been investigated by Yonge (1926, *a* and *b*), Takatsuki (1934), and others. Yonge believes that amoebocytes and digestive diverticula play an exclusive role in fat ingestion and digestion and that no fat is digested extracellularly in the stomach. Thus,

lipid droplets present in amoebocytes may represent ingested fat globules. Another interpretation is that of Gatenby and Hill (1934), who regard similar sudanophil droplets in *Helix* amoebocytes as elements of the Golgi apparatus. The perinuclear position frequently assumed by the sudanophil droplets in *Venus* amoebocytes might suggest a similar interpretation.

Neutral red vacuoles in amoebocytes. In addition to specific granules and lipid droplets, amoebocytes contained large numbers of various sizes, cytoplasmic inclusions which were stained by neutral red. That these inclusions were not the same as the specific granules stained by Janus green B was indicated by their lack of uniformity of size and shape. Also the large numbers of granules staining with Janus green B precluded the possibility that both these granules and neutral red bodies could both be present in the cell as preformed inclusions. Furthermore, it is generally agreed that supravital Janus green B rarely, if ever, stains structures which are stained by neutral red. Bensley (1911) observed that Janus green B stained mitochondria in acinar cells of the guinea-pig pancreas, whereas neutral red stained granules of prozymogen and zymogen. Gatenby (1931) stated that many cells collect and aggregate neutral red into vacuoles while it is passing through the cytoplasm, and Gatenby and Hill (1934) concluded that the neutral red inclusions of *Helix* amoebocytes were not pre-existent structures. In the case of *Venus* amoebocytes, the objects which stained with neutral red appear to be vacuoles filled with dye, rather than preformed cytoplasmic inclusions.

Pigment granules in amoebocytes. Amoebocytes frequently contained highly refractile yellow granules which appear to be identical with the excretory pigment described by Yonge (1923, 1926, *a* and *b*) and others in various lamellibranchs. These pigment granules were easily distinguished from the specific granules of these cells. Larger granules and clumps of this material were also found scattered throughout the intestinal and cardiac musculature and in especially large masses in the intestinal epithelium. The histochemistry of the excretory pigment will be considered more fully in the discussion of the intestinal epithelium.

The nature and properties of Venus intestinal epithelium. The central importance of intracellular digestion in the amoebocytes in lamellibranch nutrition has been questioned by Nelson (1933), Mansour (1946), and Mansour-Bek (1946). These workers believe that extracellular digestion occurs in the alimentary tract of these animals. Yonge (1926*a*) states that 'no evidence of any absorption in the epithelium of the gut or any free surface in the mantle cavity, other than by the agency of phagocytes was found'. Observations made on the cytochemistry of *Venus* intestinal epithelium may contribute to this question.

Red-violet granules resembling secretory granules were observed in the distal zone of the columnar epithelial cells in sections stained by eosin and methylene blue, and this region was marked by red-violet fluorescence in ultra-violet light. The eosinophil, fluorescent granules of each cell corresponded in position with the major site of alkaline phosphatase activity. This

enzyme functions in the dephosphorylation of several organic phosphates including hexose diphosphate, nucleic acid, lecithin, and glycerophosphate. Deane and Dempsey (1945) described the localization of alkaline phosphatase in the apical zone of duodenal epithelium in several vertebrate species after incubation of sections in glycerophosphate. Alkaline phosphatase activity was also seen in the supranuclear or Golgi region of these cells. These authors concluded that their studies supported the concept that intestinal epithelium contains enzymes capable of dephosphorylating intermediate substances in normal metabolism. Kosman, Kaulbersz, and Freeman (1943) reported that alkaline phosphatase was secreted by the dog duodenum and jejunum and that it probably functioned in the digestion of monophosphoric esters of food. Lecithin phosphatase is also present in duodenal epithelium and is thought to be of importance in digestion of phospholipids (Dempsey and Deane, 1946). Furthermore, glycerophosphatase is thought to be involved in fat absorption, since glycerophosphatase is believed to be an intermediate state in the breakdown and resynthesis of neutral fat (Bloor, 1943). Thus, it appears that the alkaline phosphatase activity detectable in the intestinal epithelium of *Venus* may be associated with possible digestive and absorptive functions of these cells. Further evidence for the absorptive role of the epithelium was seen in the sudanophil droplets which were present in the distal portion of the epithelium and ketone-containing lipid which was demonstrated in the middle portion of the epithelium. Gutheil (1912) observed fat globules in the intestinal epithelium of *Anodonta* and concluded that the epithelium functions in absorption, and Yonge (1926*b*) observed fat globules in the stomach and midgut epithelium of *Ostrea* after feeding on diatoms, but concluded that the amoebocytes transmitted the fat to the epithelial cells for storage. Although Yonge (1926, *a* and *b*) has denied the existence of digestive and absorptive activity in the epithelium, the presence of eosinophil granules, sudanophil droplets, alkaline phosphatase, lipase, and serum cholinesterase suggests that these functions may be present.

The nature of the goblet cell mucus. Since the goblet cells of the intestinal epithelium were strongly basophil and retained their stainability by methylene blue in solutions buffered below pH 4 and were metachromatic as well as PAS-positive after diastase digestion, it appears that they contain typical mucus. The goblet cells were stained red by the Feulgen procedure, a colour unlike the violet stain produced in the nuclei. The basis of this atypical Feulgen reaction is obscure, since this reaction is quite specific for DNA.

The nature and properties of excretory substance. Numerous investigators have noted the presence of yellow, brown, or green pigment granules in amoebocytes and other tissues (Metschnikoff, 1884; Grobben, 1887; MacMunn, 1900; Yonge, 1926, *a* and *b*). MacMunn (1900) referred to this material as enterochlorophyll, which he believed to be a derivative of ingested chlorophyll, and Durham (1891) suggested that this pigment was an excretory product composed of degraded echinochrome. Since the appearance of the pigment varied in different cells, Durham concluded that transformation of the

pigment occurred in the amoebocytes. Similar pigments were observed by J. H. List (1890) and T. List (1902). J. H. List (1890) reported that the pigment masses were not constant and that their nature depended on the food ingested by the animal since starved animals had few pigment inclusions. Yonge (1926b) observed that globules of green and brown material appeared in amoebocytes after diatoms were ingested but disappeared during starvation. The brown colour of the pigment was attributed to ingested chlorophyll and products of chlorophyll degradation. In the amoebocytes and free in the intestinal and cardiac musculature of *Venus*, excretory pigment appeared as small yellow granules, but in the intestinal epithelium this material occurred in large yellow or yellow-brown heterogenous aggregates which were composed of pigment granules embedded in a matrix.

TABLE 1. *Comparisons of the histochemical reactions of ceroid and excretory pigment*

Test	Ceroid	Excretory pigment
Colour	Yellow	Yellow
Resistance to fat solvents	+	+
Basiphilia	+	+
Acidophilia	—	—
Sudanophilia	+	+
Acid-fast	+	+
Reduction of ferric-ferricyanide	—	+
Iron (Prussian blue)	—	—
Ultra-violet fluorescence	+	+
PAS after exposure to diastase	+	+

Ceroid-like nature of the excretory substance. The description of ceroid by Endicott and Lillie (1944) is strongly reminiscent of the excretory pigment found in amoebocytes and intestinal epithelium of *Venus*. These authors identify ceroid by the following characteristics:

1. Golden yellow colour in unstained sections.
2. Resistant to fat solvents.
3. Stained by basic dyes.
4. Not stained by acid dyes.
5. Sudanophil in paraffin sections.
6. Acid fast.
7. Reduces alkaline silver nitrate, but *not* the ferric-ferricyanide reagent.
8. Iron-negative.

In addition Lee (1950) observed that ceroid in rat and mouse livers is variably PAS-positive, and Popper, György, and Goldblatt (1944) reported that ceroid possesses bright yellow or golden brown fluorescence when examined in ultra-violet light. Table 1 compares the histochemical properties of ceroid and excretory pigment.

The excretory material appears to be very similar to ceroid as described by Endicott and Lillie (1944) and others with one exception, namely, that unlike

excretory pigment, ceroid fails to stain when exposed to the ferric-ferricyanide reagent (Endicott and Lillie, 1944). However, Pearse (1953) states that this property may be acquired during the oxidation of lipids.

The origin and nature of ceroid. Ceroid was first discovered in cirrhotic livers of nutritionally deficient rats by Lillie and his associates (1941, 1942), who believed that it was a product of abnormal metabolism. It was found that ceroid accumulation was inhibited by feeding on casein, choline, and methionine. Subsequently, other workers concluded that ceroid was a lipoprotein derived from the necrotic remnants of liver parenchymal cells (György and Goldblatt, 1942; Lee, 1950), and that its presence was due to vitamin E deficiency (Victor and Pappenheimer, 1945). This pigment is found in many vertebrate tissues other than cirrhotic liver (Firminger, 1952; Deane and Fawcett, 1952; Lillie, 1941, 1942; Wolf and Pappenheimer, 1945).

Ceroid is believed to be a mixture of several substances (Lee, 1950; Pearse, 1953). Pearse (1953) regards ceroid as a member of a general group of pigments termed lipofuscins which are derived by oxidation from lipids or lipoproteins. Different staining reactions are given as the lipoidal material becomes progressively oxidized. The PAS-reaction is positive in the intermediate stages of the oxidation of lipids and Schiff-reacting aldehydes are produced from unsaturated phosphatides by periodic acid.

Casselman (1951) observed the formation of ceroid-like substances *in vitro* from unsaturated fat and from fatty acids and their esters, but never from saturated fats or hydrocarbons. The production of the pigment was prevented by the presence of antioxidants such as α -tocopherol and hydroquinone. Casselman concluded that 'whenever conditions are such that unsaturated fats accumulate in tissues to such an extent that a relative lack of biological antioxidant results, autoxidation of the fats and their conversion to ceroid pigment are favored, and that ceroid and the lipofuscin pigment of vitamin E deficiency may be fundamentally similar'.

The occurrence of a ceroid-like pigment in the amoebocytes and intestine of *Venus* was unexpected. The excretory pigment appears to be a lipofuscin closely related to ceroid observed in cirrhotic and vitamin E deficient animals. This material is formed either as an oxidized by-product of lipid metabolism in the intestine or as a by-product of intracellular digestion in the amoebocytes. The relation of excretory pigment to digestion is indicated by the disappearance of this pigment in starved animals. In the case of *Venus*, the excretory pigment may arise in part from the oxidation of phospholipids which can be demonstrated within the masses of excretory pigment.

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Lipo-Protein Cells in the Blood of *Carcinus maenas*, and their Cycle of Activity Correlated with the Moults

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SUMMARY

A description is given of cells termed lipo-protein cells in the blood of *Carcinus maenas*, and of their development from amoebocytes. These cells exhibit a marked cycle of appearance and disappearance correlated with the moult. They appear towards the end of the instar, reach their maximum development immediately before the epicuticle of the new cuticle is secreted, and disappear soon after the moult. They show positive reactions for the presence of lipids, aromatic substances, tyrosine, and peptide linkages. As the epicuticle and pre-exuvial endocuticle are secreted, the colouring with Sudan black B and the Millon reaction in the cells decrease in intensity. The cells are believed to synthesize lipo-protein or extract it from the haemolymph, and to transport it to the epidermis and thence to the developing cuticle, thus contributing to the secretion of the epicuticle and impregnating the pre-exuvial endocuticle. The lipo-protein cells are compared with the oenocytes of insects and with the granulocytes and vacuolar cells or leberidocytes of spiders.

INTRODUCTION

OENOCTES have been described in a number of insects (for review, see Richards, 1951). These large cells, which originate in the epidermal epithelium (hypodermis), have been stated in many cases to exhibit a cyclical activity correlated with the moult cycle. Wigglesworth (1933, 1947, 1948) suggests that their secretion contributes to the formation of the cuticle. In spiders (Millot, 1926; Browning, 1942), elements of the blood, the granulocytes, have been described as showing similar cyclical activity. The granulocytes contain phenolic substances (Millot and Jonnart, 1933) and reach the peak of their activity just before the new cuticle is laid down. They penetrate the epidermis and are presumed to secrete the non-chitinous substances which impregnate the pre-exuvial part of the cuticle (Browning, 1942). Other elements of the blood of spiders, the 'leberidocytes' (Deevey, 1941; Browning, 1942) or 'vacuolar cells' (Millot, 1926) show cyclical activity, the peak of which occurs at the time of moulting after part of the cuticle has been secreted. They have been considered responsible for the secretion of the chitin fraction of the cuticle (Deevey, 1941).

In the Crustacea, Kollmann (1908) has described a decline in the number of granular amoebocytes in the blood before the moult. This is attributed to the effects of enforced fasting, and is followed by an increase in the number of clear amoebocytes after the moult. Apart from this, there seems to be no record of elements with a cyclical activity correlated with the moult in Crustacea, although chemical changes in the blood have been described (Pinhey, 1930; Stott, 1932; Dennell, 1947; Krishnan, 1950). The object of this communication is to record the occurrence of lipo-protein cells in the blood of the

74 Sewell—*Lipo-Protein Cells in the Blood of Carcinus maenas*, and their decapod Crustacean *Carcinus maenas*. They originate from the blood corpuscles and show a marked cyclical activity correlated with the moult cycle. They are comparable in several ways with the oenocytes of insects and the granulocytes and vacuolar cells or leberidocytes of spiders.

MATERIALS AND METHODS

The individuals of *C. maenas* used in this study were collected at Roscoff and Plymouth. Only individuals whose greatest width (L) lay between 15 and 28 mm. were used. All stages of the moult and intermoult cycle were examined,

TABLE I

The periods of the intermoult and their subdivisions in Decapod Crustacea
(Drach, 1939, 1944)

Period	General characters of period	Phase	General characters of phase
A	The period which follows the moult. The crab is immobile.	A ₁	Starts when the crab emerges from the exuvium, with the epicuticle and pre-exuvial endocuticle already secreted. Cuticle soft.
		A ₂	Calcification of the pre-exuvial layers begins and the cuticle has a parchment-like consistency. Secretion of the principal layer of the endocuticle begins.
B	The carapace is rigid in places, the ventral parts are still soft. A period of enforced fasting.	B ₁	Distal joints (carpopodite and propodite) of the chelae are supple.
		B ₂	Distal joints of the chelae are rigid.
C	Whole skeleton is hard and the crab feeds freely.	C ₁ -C ₃	Skeleton gradually acquires complete rigidity, and the principal layer of the cuticle continues to be secreted.
		C ₄	Membranous uncalcified layer of the cuticle is secreted. This is the longest part of the cycle.
D	Part of the old cuticle absorbed and secretion of the new one begins. Enforced period of fasting begins and activity is reduced.	D ₁	Uncalcified membranous layer attacked and the new setae are formed (D ₁ '-D ₁ ''').
		D ₂	Secretion of the new cuticle begins, the epicuticle being secreted first (D ₂ ') and then the pre-exuvial endocuticle (D ₂ '').
		D ₃	Considerable absorption along the epimeral suture.
		D ₄	Cuticle splits along the suture to allow the crab to make its exit.

M O U L T

and in this account the stages referred to are those enumerated by Drach (1939, 1944). Drach divides the intermoult into four principal periods, A, B, C, and D of unequal length. These are further subdivided, and for convenience are tabulated here (table 1).

The details of identification of the different periods of the intermoult and their phases were explained and demonstrated to me by Professor Drach of the Laboratoire de Zoologie, University of Paris, during my tenure of a Fellowship awarded by the Centre National de la Recherche Scientifique.

Some of the histochemical tests mentioned in the text were carried out on fresh material, but the majority were applied to frozen sections of material fixed in 5 per cent. formalin in 0.75 per cent. saline and embedded in 25 per cent. gelatin. This initial treatment appeared to have no significant effect on the results of the tests quoted. Some histochemical reactions were effected on paraffin sections.

For histological examination, paraffin sections of material fixed in Gilson's mercurio-nitric mixture, Flemming without acetic, Carnoy's fluid, alcoholic Bouin, Pampell, and Helly were used. Gilson, alcoholic Bouin, Flemming without acetic, and Pampell proved to be the best fixatives. The material was embedded by the Peterfi double-embedding method (methyl benzoate in celloidin and paraffin wax; see Pantin, 1948). The sections were subsequently stained with Heidenhain's haematoxylin alone or with eosin, with Mallory's triple stain, and with Masson's trichrome stain.

THE HISTOLOGY AND CYCLICAL ACTIVITY OF THE LIPO-PROTEIN CELLS

No detailed account is given here of the usual cells in the blood of *Carcinus*, but they appear to be basically of two kinds—amoebocytes with few granules or with none, and granular corpuscles or granulocytes containing large numbers of uniform refringent granules in their cytoplasm. The amoebocytes are usually oval or rounded in histological preparations and amoeboid in fresh blood. Their average diameter is about 7μ (fig. 1). The granulocytes (fig. 1) are more or less rounded or oval, and are slightly larger, varying between 8 and 10μ . Both kinds of blood-cell are present at all periods.

The lipo-protein cells now to be described are easily distinguishable from the amoebocytes and granulocytes on account of their relatively large size. They are oval or rounded in both fresh and fixed preparations and are usually about 30μ in diameter when fully formed, but numbers of them are as much as 45μ in diameter. They appear in large numbers towards the end of the instar in phase C_4 , before any of the new cuticle of the following instar has been deposited but after the secretion of the whole of the membranous uncalcified layer of the old cuticle. They disappear after the moult during period B and in the later stages of the instar are completely lacking.

Sections of material fixed in the later part of phase C_4 and throughout period D show large numbers of these cells in the haemocoel, but they appear to congregate underneath the connective tissue directly beneath the epidermis

and beneath the epidermis itself, so that when the newly deposited carapace is lifted from the rest of the body, the lipo-protein cells are seen to form a dense carpet adhering to it. They are also found in large numbers between the tubules of the hepatopancreas.

They arise from ordinary amoebocytes before the cell limits of the epidermis become distinct. The amoebocytes increase in number by mitotic division in the haemolymph. Those which are to form the lipo-protein elements undergo a change in appearance and chemical constitution. They begin to

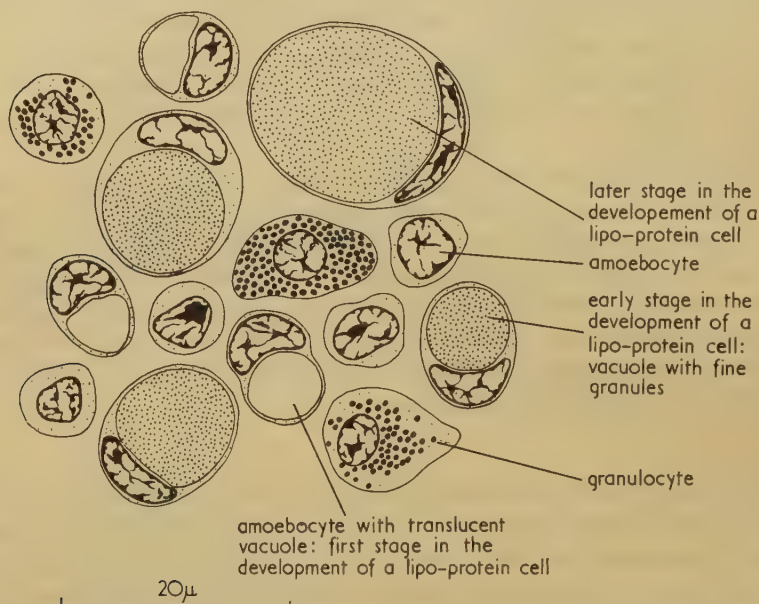


FIG. 1. Blood corpuscles and stages in the development of the lipo-protein cells seen in the haemolymph beneath the carapace epithelium and between the tubules of the hepatopancreas in *Carcinus maenas* in phase C_4 . Drawings made from paraffin sections of material fixed in Flemming without acetic and stained with Masson's trichrome stain. (L = 19.)

increase in size and as they do so a translucent vacuole appears in the central part of the cytoplasm (fig. 1). The contents of the vacuole become finely granular and as the vacuole increases in size the nucleus changes its position from a more or less central to a peripheral one and the clear cytoplasm comes to form a thin envelope around the vacuole and nucleus (fig. 1). The cytoplasm of the amoebocytes stains with eosin, light green, or aniline blue, but the finely granular portion of the developing lipo-protein cells becomes progressively more basiphil. At the same time this finely granular material begins to colour with Sudan black B and becomes positive to the Millon test. All these stages are present at one time in phase C_4 . By the end of phase C_4 and certainly by the beginning of phase D_1 the majority of the cells are fully formed. The nucleus is much flattened on the periphery of the cytoplasm, and the finely granular material has become aggregated into variously shaped pale

amber refractile bodies, $2\ \mu$ to $12\ \mu$ in diameter, which pack the cell (fig. 2) and stain intensely with haematoxylin. So densely packed are the majority of the cells with these bodies that they stain uniformly with the haematoxylin and the acid fuchsin of Mallory's triple stain, though some of the cells show the refractile bodies staining individually in the cytoplasm, which stains with eosin or aniline blue.

In late C_4 and the early stages of period D, the cells colour intensely with Sudan black B (fig. 3). This indicates that lipids are present. Furthermore,

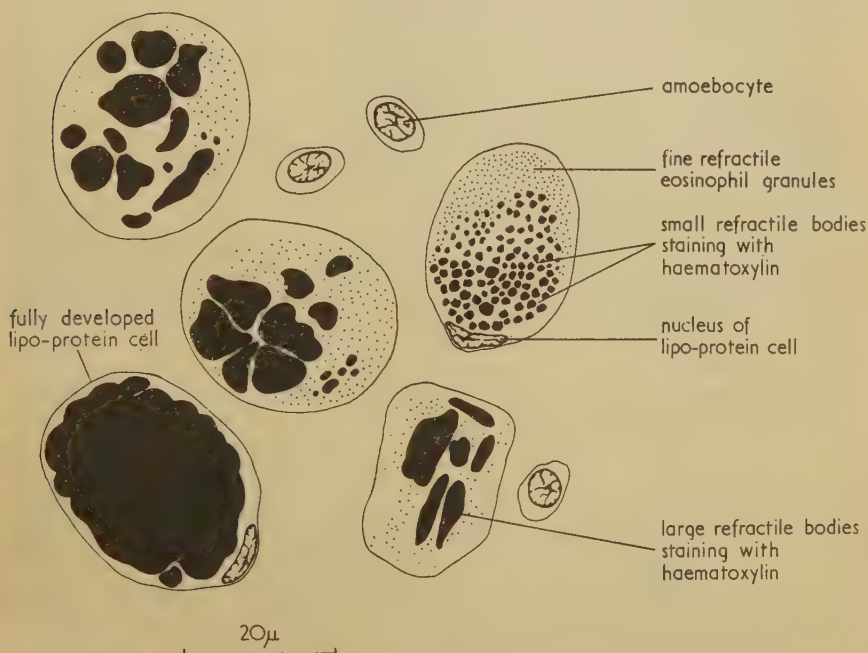


FIG. 2. Lipo-protein cells and amoebocytes from beneath the carapace epithelium and between the tubules of the hepatopancreas in *Carcinus maenas* in subphase D_1''' . Drawings made from paraffin sections of material fixed with alcoholic Bouin and stained with Heidenhain's haematoxylin and eosin. (L = 22.)

they are weakly but definitely positive to the Liebermann-Burchardt reaction, suggesting that lipids of the sterol type are present. In these stages also the cells give an intensely positive Millon reaction, a positive biuret reaction, and a weakly positive Mörner's reaction. The positive reaction to the Millon test and the colouring with Sudan black are most intense in the rounded inclusions. The cells do not reduce ammoniacal silver nitrate (the argentaffin reaction).

These reactions indicate that the cells contain in addition to lipid material a substance possessing peptide linkages and showing phenolic characters probably owing to the presence of tyrosine. The presence of a protein is strongly suggested.

During phases D_2 - D_4 the epicuticle and the pre-exuvial layer of the

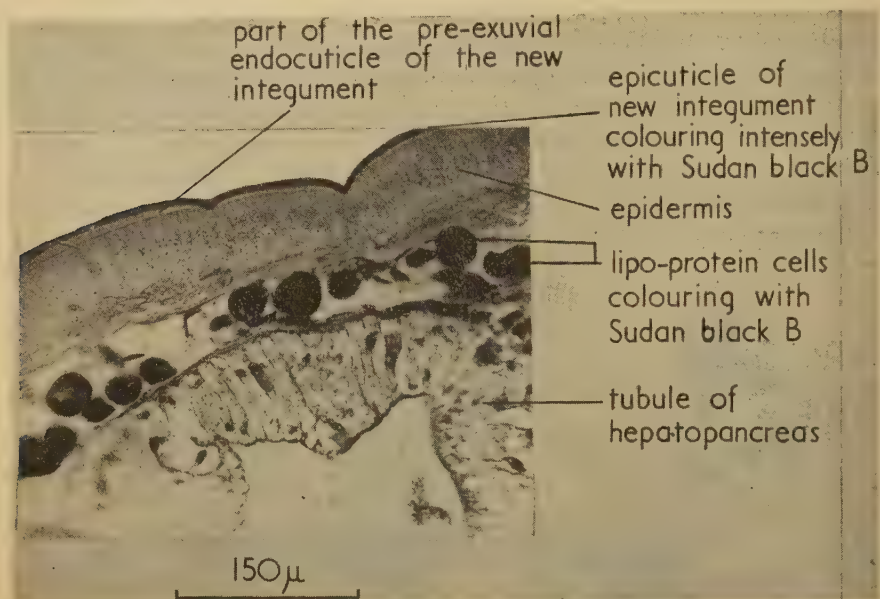


FIG. 3. Photograph of a frontal section through part of the carapace and underlying tissues of *Carcinus maenas* in the region of the hepatopancreas. Material fixed with Gilson's mercuronitric mixture early in subphase D_2'' . The old cuticle was removed before fixation. Preparation coloured with Sudan black B and mounted in glycerin jelly. (L = 27.)

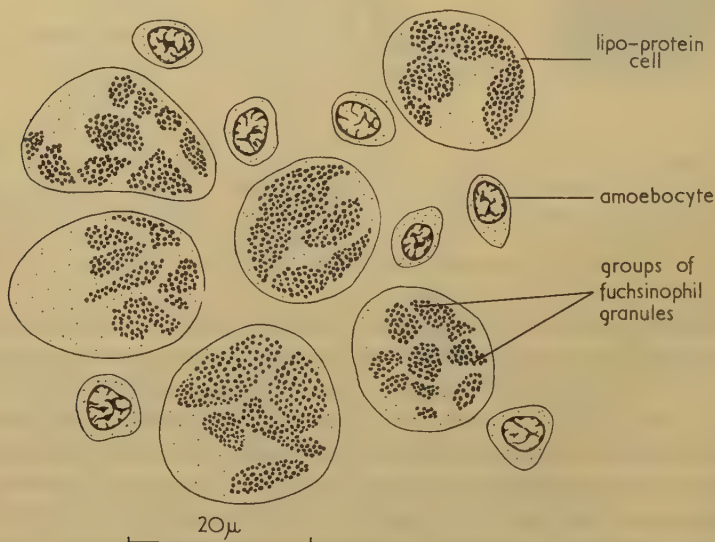


FIG. 4. Lipo-protein cells and amoebocytes from beneath the carapace epithelium of *Carcinus maenas* in phases D_3 – D_4 , showing the reformation of granules within the lipo-protein cells. Drawings from paraffin sections of material fixed in alcoholic Bouin and stained with Mallory's triple stain. (L = 27.)

endocuticle are deposited. Both these layers give positive Sudan black B, Liebermann-Burchardt, Millon, and Mörner's reactions, and are also argentaffin. It is striking that these reactions, apart from the argentaffin reaction, are the reactions given by the cells. This leads immediately to the suspicion that the cells may be involved in the synthesis or transport of material utilized in the formation of the cuticle. This suspicion is strengthened by the fact that during the period when the reactions appear in the cuticle, the intensity of the reactions in the cells begins to decline. The epicuticle seems to be deposited at the surface of the epidermal cells by direct secretion as a lipo-protein mixture; on the other hand, the lipo-protein of the pre-exuvial endocuticle appears to be secreted into this layer through the distal ends of the pore canals.

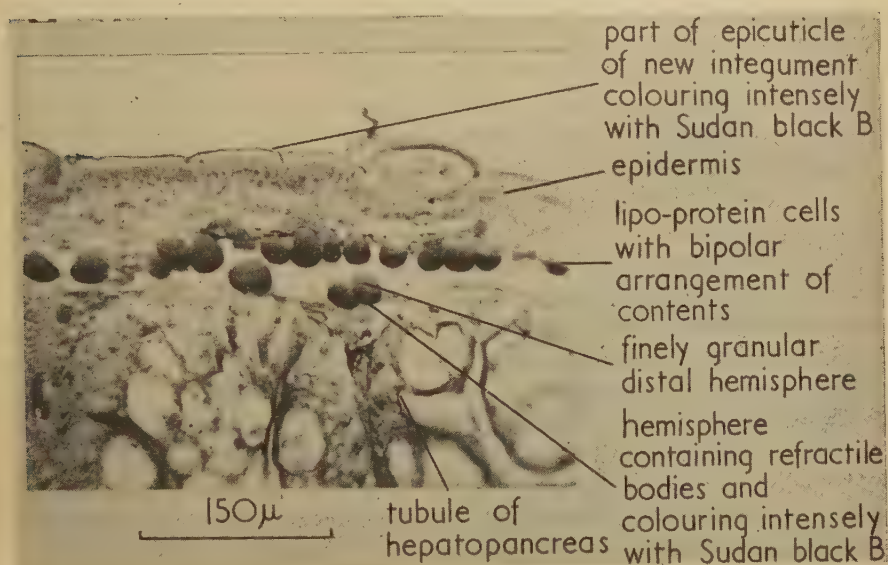


FIG. 5. Photograph of a frontal section through part of the carapace and the underlying tissues of *Carcinus maenas* in the region of the hepatopancreas. Material fixed with Pampell's fluid in subphase D_2' and showing the bipolar arrangement of the contents of the lipo-protein cells. The old cuticle was removed before fixation. Preparation coloured with Sudan black B and mounted in glycerin jelly. (L = 15.)

With the diminution in intensity of the Millon reaction and of Sudan colouring in the cells as the moult is approached, the contents become once more finely granular. They no longer stain with haematoxylin or acid fuchsin and only slightly with light green or aniline blue. The appearance of fine granules is evident in phases D_2 and D_3 . The refractile bodies which stained so conspicuously with the acid fuchsin of Mallory's triple stain during the earlier stages of their development, appear to disintegrate, and instead of fuchsinophil bodies, variously shaped groups of fuchsinophil granules are observed within the cells (fig. 4). These granules eventually stain bluish grey in Mallory preparations, but after the moult their distribution is more or less homogeneous

throughout the cell. After the moult the number of cells diminishes and towards the end of period B they have disappeared. Whether they disintegrate and liberate their granules into the blood stream or whether, as seems possible, they revert to amoebocytes, has not been determined.

In phase D₂, there was observed in some preparations a pronounced tendency for the refractile bodies to congregate in the hemisphere of the cell farther from the epidermis, leaving a finely granular cytoplasm in the other hemisphere. This distribution of the cell contents leads to a most curious and striking bipolar appearance on staining and the application of certain histochemical tests (fig. 5). With Mallory's triple stain the hemisphere containing the refractile bodies stained vividly with acid fuchsin and the hemisphere nearer the epidermis, in marked contrast, with aniline blue. The hemisphere containing the refractile bodies also gave markedly more intense Millon and Sudan black B colouring than the other hemisphere. This bipolar effect was never observed with certainty in fresh material, but it would seem to result from some real polarity in the cell, since the orientation always bears the same relation to the epidermis secreting the new cuticle.

DISCUSSION

It has been shown (Dennell, 1947; Krishnan, 1951) that the Crustacean cuticle, in addition to being hardened with calcium, undergoes phenolic tanning in the manner characteristic of insects to a slight extent. In insects the cuticle becomes impregnated by a sterol and a protein rich in tyrosine, and this is the substrate which becomes tanned (Malek, 1952). It may therefore be justifiable to expect that the Crustacean cuticle is similarly impregnated. This expectation is realized by the observation in the present work, that the cuticle of *Carcinus* contains a lipid which gives a positive Liebermann-Burchardt reaction, and a protein containing an aromatic nucleus.

In *Carcinus* the cycle of appearance and disappearance of the lipo-protein cells, so closely correlated with the deposition and lipo-protein impregnation of the pre-exuvial layers, leads to the belief that the cells have some definite role in the formation of these layers. It would appear that they synthesize or extract from the haemolymph and transport to the epidermis, the lipo-protein which subsequently impregnates the developing cuticle.

The lipo-protein or its precursors which the lipo-protein cells elaborate from the haemolymph must have its ultimate origin in the food and it is interesting to note that in *Cancer pagurus* the reserves of protein, lipid, and glycogen in the hepatopancreas increase in the period in which the animal feeds (Renaud, 1949). These reserves reach a maximum in phase C₄ and the beginning of subphase D₁', and then begin to decrease. Renaud observes that this decrease is due to the fact that a part of the reserves, especially the proteins and glycerides, are used up for the subsistence of the animal during the period of enforced fasting which starts at the beginning of period D₁ and to the fact that another part (the esters of cholesterol, 'unsaponifiable X', some of the

proteins, and finally the glycogen) is directed towards the epidermis for the formation of the cuticle. Now, the lipo-protein cells of *Carcinus maenas* make their appearance towards the end of phase C_4 and reach their maximum development at the end of phase C_4 and the beginning of phase D_1 . This coincides approximately with the decline in the lipid and protein content of the hepatopancreas in *Cancer pagurus*.

Renaud (1949) also records a marked increase in the lipid content of the epidermis of *Cancer pagurus* at the end of subphase D_1'' and the beginning of subphase D_1''' , that is, immediately before the epicuticle is secreted. At these stages the lipid represents 33 per cent. of the dry weight of the epidermis as compared with 19 per cent. of the dry weight in phase C_4 and 23 per cent. in subphase D_2'' , when the epicuticle is complete. This increase in the lipid content of the epidermis of *Cancer pagurus* coincides with the decline in colouring by Sudan black that is observed in the lipo-protein cells of *Carcinus maenas* during the course of the present work.

The function of the lipo-protein cells of *Carcinus maenas* would seem to correspond closely with that of the oenocytes of insects (for review see Richards, 1951; Wigglesworth, 1933, 1947, 1948; Kramer and Wigglesworth, 1950) in synthesizing some of the non-chitinous constituents of the cuticle. Both elements reach the peak of their activity just before the epicuticle of the new cuticle is laid down and then gradually diminish or disappear after the moult. However, the lipo-protein cells and the oenocytes differ fundamentally in their origin. As already described, the lipo-protein cells of *Carcinus* originate from blood corpuscles whereas the oenocytes of insects originate from epithelial cells (Wigglesworth, 1933), a new generation arising at each moult. The lipo-protein cells retain a more or less spherical form but the oenocytes are sometimes lobulated (Wigglesworth, 1933), the lobes becoming detached to form a new generation of oenocytes (Wigglesworth, 1947). The lipo-protein cells of *Carcinus* do not penetrate the epidermis even though they become closely applied to its base, and their secretion can only reach the cuticle through the epidermal cells. The oenocytes, on the other hand, penetrate the epithelium and considerably attenuate its cells. Even so it would seem that the products of their secretion do not pass directly into the cuticle but reach it by way of cytoplasmic processes of the attenuated epithelial cells (Kramer and Wigglesworth, 1950). The lipo-protein cells of *Carcinus*, once the pre-exuvial layers of the cuticle have been secreted, begin to disappear and are completely absent from the middle stages of the instar. The oenocytes, despite the fact that their activity reaches a peak just prior to the moult, are present throughout the instar and in the adult female of *Rhodnius* reach another peak of activity during the formation of the lipo-protein egg-cases (Wigglesworth, 1933).

However, if in fact the lipo-protein cells, arising as they do from amoebocytes, revert to amoebocytes after the moult, then the lipo-protein cells cannot truly be regarded as transitory *ad hoc* structures present only at the time of moulting, but rather as a phase of activity of the amoebocytes with a peak just

before moulting. The cycle would then correspond more closely with the oenocyte cycle, despite the difference in origin.

In spiders (Millot, 1926; Browning, 1942), as in *Carcinus*, certain blood corpuscles, the granulocytes, appear to be at least partly responsible for the secretion of the exocuticle. These corpuscles are packed with granules and like the oenocytes of insects, but unlike the lipo-protein cells of *Carcinus*, penetrate the epithelium during the secretion of the pre-exuvial part of the cuticle. They similarly reach the peak of their activity before the secretion of the new cuticle begins. Like the oenocytes of insects, they are present throughout the instar though their number declines between moults.

However, there are other blood corpuscles, the vacuolar cells (Millot, 1926) or leberidocytes (Deevey, 1941; Browning, 1942), which are only present during the period of moulting, but reach the peak of their activity at the time of the actual moult or directly after it, that is, after the secretion of part of the new cuticle. These are most interesting in the present context in that they arise from ordinary leucocytes in exactly the same way as do the lipo-protein cells of *Carcinus* from the clear amoebocytes. That is, a vacuole is formed within the corpuscle and gradually increases in size, confining the cytoplasm to the periphery and displacing the nucleus from the centre and flattening it at one pole. However, the leberidocytes do not appear to produce the large refractile bodies as seen in the lipo-protein cells of *Carcinus*, nor does there appear to be any record of phenolic or proteic contents. Browning (1942) observes that the contents of the vacuole are fluid but in *Carcinus* they are at first granular and later definitely solid. The difference in time of the peak of their activity would suggest that their role is not identical. Here it is held that the lipo-protein cells of *Carcinus* provide the lipo-protein which impregnates the pre-exuvial layers of the cuticle. Deevey (1941) suggests that the leberidocytes of spiders secrete the chitin fraction of the cuticle in view of the presence of glycogen within them.

Thus it would not seem feasible to call the lipo-protein cells of *Carcinus* either oenocytes, in view of their origin, or leberidocytes, and at this stage the term lipo-protein cell seems an adequate name to give them.

This work was begun at the Station Biologique de Roscoff, and continued in the Department of Zoology, Manchester University. I wish to thank Professor H. Graham Cannon for his suggestions and Professor R. Dennell for his help and advice. I am greatly indebted to Professor P. Drach of the Laboratoire de Zoologie, University of Paris, for his help and for instructing me in his admirable technique for determining the intermoult stages of Crustacea, which is indispensable in a study of this kind.

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Experiments on the Mechanism of Silver Staining

Part I. Impregnation

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With 1 plate (fig. 3)

SUMMARY

In the impregnating stage of silver staining, there are essentially two different reactions taking place between the silver ions and the sections of fixed tissue. A substantial part of the silver is combined with the tissue in the unreduced form, and a smaller part is reduced to produce silver nuclei which form centres on which the developing agents can deposit additional silver derived from the combined (reducible) silver fraction.

A study of the effect of pH and time of impregnation, as well as of the specific blocking of chemically active protein end-groups upon the reducible silver fraction, suggests that this is chiefly combined with histidine and that it is not limited to the sites of the final differential staining. Except for the cell nuclei, the reducible silver shows a uniform distribution throughout the section; the amount taken up is in equilibrium with the silver ion concentration in the impregnating bath.

The fraction present as silver nuclei increases progressively with time, with increasing pH, and with the temperature of the impregnating bath. Silver ions appear to be converted to silver nuclei by a process of physical reduction, and once formed, the nuclei are quite stable.

Deposition of silver nuclei, necessary for differentiation of the stain, only occurs after impregnation between pH 7.5 and 9.0. In addition, it is only within this range that a sufficient amount of reducible silver is taken up to produce deep staining after development.

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I. INTRODUCTION

THE silver staining methods are very important for the study of nervous tissue but, as with many other methods of histological staining, the methods used are generally empirical, and the principles involved little understood. The present work is an attempt to further an understanding of these principles.

The first theoretical approach to the subject was that made by Liesegang (1911), who postulated that during the impregnation of the tissue in the silver solution, nuclei of reduced silver are produced, which, on development of the stain, act as centres for the further deposition of silver reduced by the developing agent, with the result that a visible staining is produced. This theory involves many parallels with photography, and the silver nuclei may be likened to the latent image produced on exposure of a photographic emulsion. Liesegang's theory has formed the basis of many later theories on silver staining, and has been greatly extended by the work of Silver (1942), Holmes (1943), Palmgren (1948), Romanes (1950), and Samuel (1953*a, b*). The recent work on silver staining has confirmed that there are two forms of silver contained in impregnated nervous tissue, namely, silver which has been reduced to form nuclei, and developable silver which is combined with the components of the tissue. The developable silver is reduced by the developer and probably deposited in relation to the silver nuclei.

II. METHOD OF STAINING

Before either the factors affecting silver staining or the mechanism of staining could be investigated, it was necessary to evolve a simple method of staining which could be consistently repeated. The techniques generally used for silver staining of paraffin sections are complex, and the reagents variable; especially when the method involves the use of protargol or other silver proteinate. However, three recent techniques, namely, those of Holmes (1943 and 1947), Romanes (1950), and Samuel (1953*b*), have made use of dilute solutions of simple silver salts. Holmes used a 1/10,000–1/100,000 solution of silver nitrate buffered at pH 7.8 or 8.4 by boric acid/borax buffer, and Samuel a modification of Holmes's technique employing a 1/10,000 solution of silver nitrate. Romanes used a 1/30,000 solution of silver chloride, the pH of which was adjusted by ammonia to 7.8, as indicated by comparator papers; this is equivalent to pH 9 as shown by a glass electrode (Romanes, personal communication). I found, as did Romanes, that the optimum pH for impregnation before chemical development was pH 9, and unless otherwise stated the following method of staining was employed throughout the present work:

- (1) Place pieces of fresh tissue in 70 per cent. alcohol for a sufficient time to allow fixation, dehydrate in alcohol, and embed in paraffin wax.
- (2) Mount paraffin sections on slide with albumen; remove the paraffin wax.

(3) Impregnate in the following solution in an incubator at 37° C. for 16 hours: 1 ml. of 1 per cent. silver nitrate, 180 ml. of distilled water, and 20 ml. of 0.1 M boric acid/borax buffer at pH 9 (final concentration of silver nitrate is 1/20,000). A standard buffer of pH 9 is made up by mixing solutions of 0.1 M borax and 0.1 M boric acid until the pH value, as indicated by the glass electrode, is 9.

(4) Transfer the slides to the developing solution, after rinsing in distilled water.

(5) Wash in tap water for 10 minutes.

(6) If toning is required, pass through distilled water to 0.2 per cent. gold chloride for 3 minutes.

(7) Transfer to 2 per cent. oxalic acid for 2 minutes.

(8) Wash in tap water.

(9) Transfer to 5 per cent. sodium thiosulphate for 10 minutes.

(10) Wash well in tap water, followed by distilled water, dehydrate, and mount.

III. COMBINATION OF SILVER WITH THE TISSUE

To understand the mechanism of silver staining it is clearly necessary to determine how the silver, which can be subsequently reduced by the developer, is combined with the sections. It seems unlikely that the silver ions will combine with the fats and carbohydrates to any great extent, and therefore only the proteins will be considered. Recent work on the combination of metal ions with proteins suggests that the combination may be stoichiometric (Klotz, 1952), and the following investigation appears to support this view.

(a) *Method*

The tissues used in this investigation consisted of transverse sections of frog spinal cord, frog sciatic nerve, and rat cerebellum. These had been fixed in alcohol, which is assumed not to combine with the proteins to any extent during fixation. After treatment, the sections were stained by the method previously outlined.

(b) *The effect of pH*

The pH of the impregnating solution in these experiments was adjusted from pH 4.5 to 5.5 by acetate-acetic acid buffers, and over the range pH 6.3 to 9.0 by boric acid/borax buffers. The final concentration of the buffer in each case was 0.01 M. Using silver electrodes, it was found that the concentration of free ionic silver in the impregnating solution was fairly constant over the whole of the pH range used.

After development with a hydroquinone-sulphite developer, there was a deeper staining at the higher pH values; this agreed with the results of Romanes (1950) and Samuel (1953*b*). There was little staining below pH 7, although there was a rapid increase in the intensity of staining up to pH 9. If, however, the silver combined with the section was precipitated by more

active agents, such as an acid solution of hydrogen sulphide or a 2 per cent. solution of hydroquinone, which precipitate the silver more rapidly than the sulphite-containing developing solutions, there was some precipitate on the sections below pH 7. The amount of silver precipitated after impregnation at pH values below pH 7 was small, but these experiments showed that with an increase in the pH value the combined silver increased in a much more uniform manner than the results with the developer had suggested.

The marked increase in the depth of staining above pH 7, which occurred after development with the hydroquinone-sulphite solution, might suggest that the silver combines with the carboxyl groups, which would be expected to ionize at pH 5 to 6. A more probable explanation is that below pH 7 there were not enough silver nuclei formed in the section during impregnation to act as centres for the reduction of the combined silver by the developer. The precipitation of the silver by more active reagents suggests that the combination of the silver with the section involves some weak group rather than the carboxyl groups.

(c) *The effect of duration of impregnation*

The effect of time on the silver uptake was determined at pH 9. Sections were impregnated, rapidly rinsed in distilled water, and, as above, immersed in either an acid solution of hydrogen sulphide or a 2 per cent. solution of hydroquinone. At 37° C. the total uptake of silver by the section appeared to be almost complete at the end of 10 minutes, although there was a slight increase in the amount combined up to 30 minutes. At 19° C. the rate of combination with the silver was slower, but was almost complete at the end of 30 minutes.

When similar sections were developed in the hydroquinone-sulphite developer, staining was only slight up to 1 hour, but was almost complete at the end of 2 hours; there was very little further increase in the density of staining up to 17 hours, although there was an improvement in the details of staining. Samuel (1953*b*), also using a hydroquinone-sulphite developer, found that at 56° C. staining occurred at pH 9.1 after 15 minutes' impregnation, and that good staining was obtained at pH 6.8 after 2½ hours' impregnation. In none of the present experiments was the staining so rapid.

Thus the density of *differential* staining obtained by the use of a normal developing solution takes about 2 hours to reach an optimum. This is probably due to the relatively longer time necessary for the formation of a sufficient number of silver nuclei to produce normal development, because the experiments with more active reagents showed that the combination of reducible silver with the section was almost complete at the end of 10 minutes.

(d) *The effect of the concentration of silver*

Sections were impregnated in solutions of 1/20,000, 1/40,000, 1/100,000, and 1/200,000 silver nitrate for 3 hours at pH 9. The combined silver was deposited by immersing the sections in either a 2 per cent. solution of hydro-

quinone (fig. 3, H) or an acid solution of hydrogen sulphide. The results showed that the amount of reducible silver combined with the section increases with an increased concentration of silver in the impregnating bath. There is no question of a removal of all the silver ions from the solution; this result therefore means that there is an equilibrium between the silver ions in the impregnating bath and the silver combined with the section. This suggests that the combination of silver with the section is reversible and probably due to a weak grouping; otherwise, provided that there are sufficient silver ions in the impregnating bath, the amount of silver combined with the section would be expected to be constant.

(e) *The effect of blocking of protein and other end-groups*

The above experiments gave some indication of the type of combination taking place between the silver ions and the section, but to determine more specifically which amino-acid was involved in the combination, the end-groups of the amino-acids in the sections were treated with specific reagents before impregnation. The effect that the end-group reagents had on the impregnation was used to determine which amino-acids were involved in the combination of silver with the sections.

In these experiments some of the protein reagents suggested by Olcott and Fraenkel-Conrat (1947) and Danielli (1950) were employed. The results, and the conditions used for blocking the groups, are shown in table 1, which indicates the specificity of the stain after blocking together with the intensity of the staining (see also Peters, 1953).

The results show that while the carboxyl groups appear to play a part in determining the specificity of the stain (fig. 3, B and C) they are not the groups responsible for the general non-specific uptake of silver; this is also indicated by the effect of pH. That the carboxyl groups were blocked after esterification was proved by treating the sections with methylene blue, a basic dye, which did not stain the treated sections although control sections were fully stained. The effect of diazomethane is difficult to interpret, since this compound is an active methylating agent and probably reacts with other groups as well as the carboxyl groups.

The possibility of the aldehyde groups playing a part in the reactions of the sections can be eliminated, because the compounds containing these groups would be extracted by the alcohol fixative used (Danielli, 1949); Schiff's reaction was negative on the sections. This point will be considered in more detail later. Extraction of the nucleic acids by trichloroacetic acid (Schneider, 1945) did not affect the staining apart from reducing the staining of the nucleolus.

Hydrolysis of the phosphate and sulphate esters by 1 M hydrochloric acid at 37° C. had no effect.

There remain to be considered the ring-containing amino acids—tryptophane, tyrosine, and histidine. Two reagents used were 2:4 dinitrofluorobenzene (DNFB) (Danielli, 1947), and benzoyl chloride (Danielli, 1950). Both greatly reduce the intensity of staining, DNFB to a greater extent than

benzoyl chloride. DNFB blocks tyrosine and histidine (Porter, 1950), while benzoyl chloride blocks all three ring-containing compounds. Performic acid was also used to block tryptophane but was of little use, for although it reduced the intensity of staining (fig. 3, D), a similar effect was brought about by

TABLE I
The effect of blocking agents on staining

Reagent	Conditions of blocking reaction	Specificity of staining	Intensity of im-pregnation	Refer-ence
Control		++	++	
<i>Amino-groups:</i>				
Nitrous acid	1 M NaNO ₂ —HCl; 0° C.; 2 hrs.	++	+	
Formaldehyde	Neutral fixative; 4 per cent.	++	++	
Picric acid	Fixative; saturated solution	++	++	
Iodoacetic acid	0.1 M; pH 9.6–10.6; 37° C.; 7 hrs.	++	++	(1)
<i>Carboxyl groups:</i>				
Esterification	0.1 M HCl in abs. MeOH; 37° C.; 48–65 hrs.	+	++	
Diazomethane	Ethereal solution; 18° C.; 4 hrs.	0	+	
<i>Sulphydryl groups:</i>				
Iodoacetic acid	0.1 M; pH 9.6–10.6; 37° C.; 7 hrs.	++	++	(1)
Hydrogen peroxide	0.05 M; pH 7; 18° C.; 45–72 hrs.	++	+	(2)
Periodic acid	1 per cent. aq. soln.; 18° C.; 2 hrs.	++	++	(3)
Performic acid	90 per cent. formic acid—H ₂ O ₂ ; 18° C.; 10–15 min.	+	0(+)	(4)
<i>Disulphide links:</i>				
Thioglycollic acid.	0.1 M; pH 8.4–7.7; 18° C.; 12–15 hrs.	++	++	(5)
Iodoacetic acid	0.1 M; pH 9.6–10.6; 37° C.; 7 hrs.			
<i>Ring compounds:</i>				
DNFB	0.15 M DNFB in 90 per cent. EtOH saturated with NaHCO ₃ ; 18° C.; 24 hrs.	+	+	(6)
Benzoyl chloride	10 per cent. in dry pyridine; 18° C.; 17 hrs.	+	+	(7)

(1) Michaelis and Schubert (1934).

(2) Olcott and Fraenkel-Conrat (1947).

(3) Dempsey, Singer, and Wislocki (1950).

(4) Sanger (1945).

(5) Goddard and Michaelis (1934).

(6) Danielli (1947).

(7) Danielli (1950).

formic acid alone; this acid is necessarily present in large quantities in any preparation of performic acid. Another oxidizing agent, acidified potassium

permanganate, also reduced staining; it is known to disrupt the imidazole ring of histidine (Karrer, 1947, p. 773). The reaction which takes place in the presence of formic acid is unknown. Evidence against the extensive participation of tryptophane is suggested by the fact that if frozen sections of gelatin are stained by the present method, they stain quite deeply, although gelatin contains no tryptophane.

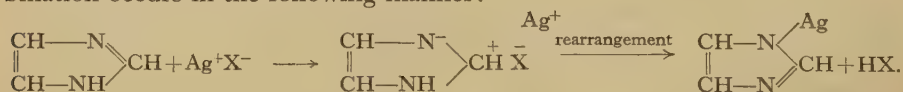
Since staining begins as low as pH 7, it suggests that the silver probably combines with the imidazole group of histidine. This has a pK_b of 7.97 (Hill and Branch, 1940); the pK value of the $-OH$ group of tyrosine is 10. Further evidence is obtained from the silver staining of smears of the following proteins:

Haemoglobin > Fibrinogen > Pepsin > Protamine.

The intensity of staining shows a descending order which is paralleled by their histidine content (Tristram, 1949).

(f) *The role of histidine in silver combination*

The results suggest that reducible silver is taken up by the tissue in combination with histidine, and this confirms the recent work of Wormser (1951), who showed that in dialysed solutions of serum-albumen the histidine was responsible for silver combination. Grassmann and Kusch (1952) also concluded that since the amount of silver combined at pH 6.2 with a series of collagen-type proteins was proportional to their histidine content, the silver combined with this amino acid. The imidazole nucleus is known to form complexes with silver and thought to combine with silver bromide grains in photographic emulsions (Mees, 1944, p. 191). Mees suggests that the combination occurs in the following manner:



He also states that there may be a combination of the silver with the guanidyl grouping of arginine, since this contains the group $-\text{CH} = \text{NH}$, which is considered to act in the same way as the imidazole nucleus of histidine:



The above results by no means exclude the possibility of combination between silver and arginine, but this could not be investigated owing to the lack of specific blocking reagents for guanidyl groups. Arginase cannot be employed because the enzyme only attacks free arginine. Staining experiments on protein smears appear to exclude the possibility of an extensive combination between silver and arginine because a smear of protamine showed little uptake of silver, though the arginine content of this protein is about 87 per cent.; furthermore, the pK value of the guanidyl group is 12–13. A further

possibility is the sulphhydryl group, for although the above experiments give only a slight suggestion of combination, the —SH group is known to form compounds with silver; the pK value is 9–11.

Gorriz (see Weber, 1947) suggested that formalin used as a fixative methylates the neurofibrils and thus mordants the tissue. The fact that methylation of the tissue either by diazomethane or methyl alcohol reduces that staining suggests that Gorriz's inference is incorrect.

M. L. Silver (1942) states that the amount of silver combined with the sections increases with time, but Romanes (1950), has shown that after a given time there is little increase in the amount of silver taken up by the sections from the impregnating bath; we have confirmed Romanes' result. Romanes attributed this to a saturation of the reducing groups in the tissue, and believed that the groups involved were the aldehyde groups. However, we have pointed out that these groups will be absent after alcohol fixation, and suggest that the groups which are combined with the reducible silver are the imidazole groups of histidine, together with any other groups which may combine with silver. Thus combination between the sections and silver is assumed to be stoichiometric and chemical; further, it has been shown that there is an equilibrium between the silver combined with the section and the silver ions in the impregnating solution.

If the combination of the silver is with histidine then, before the slides are developed, silver will not be specifically confined to the nerve elements; it would be expected that the distribution of the silver-combining groups is general. Therefore an attempt was made to determine the site of histidine in the sections, but no specific test for histidine could be found. A modified form of the Knoop test (Hunter, 1951) was attempted on the sections, but without success. In all of the colour tests available the reagents react with tyrosine or tyrosine and tryptophane. However, sections were condensed with diazotized sulphanilic acid at 0° C. for 15 minutes, washed, and mounted in glycerine. This reaction produces a yellow colour with tyrosine and histidine which, as far as could be seen, was not confined to any specific elements of the tissue.

To determine the position of silver before development, a number of slides were impregnated, washed to remove any traces of uncombined silver, and then immersed in an acid solution of hydrogen sulphide to precipitate the reducible silver *in situ*. Although the cell nuclei were blackened more than the rest of the section, there was no specific deposition of silver sulphide on the fibres, and the precipitate was fairly uniform. Therefore, before the silver is developed, it appears that the combination of the unreduced silver with the section is relatively unspecific. Since development leads to specific staining, the combined silver is not reduced *in situ* by the developer; the specificity of the silver preparation is therefore determined in the actual process of development.

IV. THE FORMATION OF SILVER NUCLEI

Holmes (1943) claimed that during the immersion of formol-fixed sections in the impregnating bath, nuclei of reduced silver are formed in them. He

impregnated paraffin sections, washed them in distilled water for 6 hours, then developed and toned with gold. He states that although the sections showed little or no darkening after development, on toning there was an almost complete staining of the tissue elements; Romanes (1950), who used no developing agent, found that the staining was both light and incomplete.

A similar experiment to that of Holmes was carried out with tissues fixed in alcohol, picric acid, chloral hydrate, and formol. Slides were impregnated and washed in several changes of distilled water for 24 hours before development in chloroquinol; hardly any staining was found after toning except a light but incomplete staining of the formol-fixed sections. This staining was probably due to a slight initial reduction of the silver in the impregnating bath. Sections from the other fixatives showed no reduction at all, which suggests that this method gives no true indication of the presence of silver nuclei in these sections.

Two types of developing solution have been used in silver staining. Those which contain free silver ions in the developing solution initially will be referred to as 'physical developers', and those which contain no free silver ions will be referred to as 'chemical developers' (see Peters, 1955). In photography the deposition of silver from a physical developing solution is controlled by the 'latent image' in the emulsion. Similarly, in sections of tissue the presence of silver nuclei should lead to a deposition of silver from such a developing solution. Thus, in physical development the developed silver is provided by the developing solution and, in the main, is not derived from the unreduced silver combined with the proteins of the section, as it is in chemical development.

Samuel (1953*b*) used the physical developer suggested by Pearson and O'Neill (1946) to demonstrate the presence of silver nuclei in impregnated sections. To remove the reducible silver he immersed the sections in a 2.5 per cent. solution of sodium sulphite for 5 minutes before development. In the present experiments, a glycine-containing physical developer was used to determine the presence of silver nuclei (Peters, 1955).

Sections which had been impregnated were washed and placed in the developing solution; in every case a complete development of the stain resulted (fig. 3, E). Consequently, sections fixed in fixatives other than formol, although they may not show any visible reduction of silver on removal from the impregnating bath, do contain silver nuclei. Sections which had not been impregnated were placed in the physical developer, but in no case was there any specific staining, and the deposition of silver on to the section was only slight.

(a) Factors affecting the formation of silver nuclei

The factors affecting the formation of silver nuclei in the impregnating bath were investigated by the use of the glycine physical developer. In these experiments the assumption is made that the depth of staining produced by the physical developer in a given time is dependent on the number of silver

nuclei in the tissue. Whether these nuclei act as centres for the catalytic reduction of silver or as centres for the deposition of silver, the greater their number the greater will be the silver deposition, provided that the conditions of development are constant. This assumption appears to be fully valid, since James (1950, p. 111) found that silver sol particles accelerate the reduction of silver ions by developing agents; the rate of reduction was proportional to the amount of silver sol added.

In all cases sections of alcohol-fixed frog spinal cord, frog sciatic nerve, and rat cerebellum were used.

(1) To determine the stability of the silver nuclei five slides were impregnated for 16 hours, then transferred to distilled water, which was changed five times during the first day and subsequently once every day. Slides were removed from the distilled water after 1, 2, 3, 5, and 7 days, and developed. The pH of the distilled water used to wash the slides was 6. Although the time necessary for development at room temperature increased from 16 to 18 minutes during the first 5 days, development was normal. After 7 days, however, 32 minutes was required for development and, in contrast to the previous slides, resulted in a very deep staining of the nerve-cell nuclei, relative to that of the nerve fibres. Nevertheless, the silver nuclei were still present in the tissue after 7 days' washing, and consequently they appear to be quite stable.

(2) Impregnation in 1/40,000, 1/20,000, 1/5,000, and 1/2,000 silver nitrate, followed by washing for 24 hours before development, showed that the rate of formation of silver nuclei increased with the concentration of silver in the impregnating bath. After impregnation in 1/2,000 silver nitrate the staining was less specific than when more dilute solutions were used.

(3) Impregnation was carried out in 1/20,000 silver nitrate at 0° C., 10° C., 14° C., and 37° C. for 16 hours and followed by washing the sections in distilled water for 24 hours. The results showed that the rate of formation of nuclei increases with temperature. Very little staining was obtained, even after 18 minutes' development, in the sections impregnated at 0° C. and 10° C., while sections stained at 37° C. gave quite normal staining.

(4) Silver nuclei increase progressively with time. They begin to appear soon after immersion of the sections in the impregnating bath. With long periods of immersion, of the order of 8 days, the reduction of silver on to the sections can be appreciated more fully. After such a period of time the sections are quite brown in colour, and many of the structural details of the sections are visible as though the section had been impregnated and developed normally, although the details are not as clear. This is probably due to an initial formation of nuclei which act as centres for the further deposition of silver from the solution, so that a very slow type of physical development occurs. The reason for the lack of detail in the sections after such a long impregnation is probably that silver nuclei are formed in other tissue elements as well as the nervous elements. A similar effect is noticed when sections are impregnated for 2 or 3 days before development; here again there is some loss of specificity of the staining.

(5) The effect of pH was investigated by impregnating the sections in a 1/20,000 solution of silver nitrate for 16 hours at 37° C. The pH of the solutions was adjusted at 4.7 and 5.5 by acetate-acetic acid buffers, and at 7.0, 8.0, and 9.0 by borate/boric acid buffers. The final concentration of the buffer was 0.01 M.

In the first experiments the reducible silver was removed from the sections by washing them in several changes of distilled water for 24 hours before development in either the glycine physical developer or in a modification of Pearson and O'Neill's developer (Samuel, 1953*b*). A similar set of experiments was also carried out in which the reducible silver was removed from the sections by immersing them in either 2.5 per cent. sodium sulphite for 5 minutes or in a citrate buffer at pH 3.2 for 1 hour before development (Samuel, 1953*b*). In all cases where the glycine physical developer was used, the deepest staining was obtained at pH 8.0, indicating that there was a maximum formation of silver nuclei at this pH value. On either side of this value the depth of staining was less, and at pH 5.5 and 4.7 only very faint and unspecific staining occurred (fig. 3, G). The staining was specific at pH 7.0, 8.0, and 9.0, but the best differentiation of fibres was found at pH 8.0.

Using Pearson and O'Neill's developer there was a maximum depth of staining at pH 8.0 when the reducible silver was removed by continuous washing, and the maximum varied between pH 7.0 and 8.0 when the reducible silver was removed by sodium sulphite treatment. Samuel (1953*b*), in experiments carried out over the range pH 6.8 to 9.1, found an optimum for the formation of nuclei at pH 6.8, and that as the pH rose the amount of silver nuclei per unit volume of the tissue decreased. With Pearson and O'Neill's developer the staining was only specific at pH 7.0 and 8.0; outside this range the staining was coarse and unspecific. A similar result was found by Samuel (1953*b*).

The results indicate an optimum formation of nuclei at pH 7–8; on each side of this range there is a decrease in the rate of formation of nuclei.

(6) Since very little staining was produced by chemical development at pH 4.5, the formation of silver nuclei in higher concentrations of silver nitrate, namely, 1/100, 1/500, 1/1,000, and 1/5,000, was investigated at this pH value. Impregnation was for 18 hours at 37° C. As in the experiments carried out at pH 9, there was an increase in the staining with concentration, so that the staining was quite deep after impregnation in the 1/100 silver nitrate. However, the staining was completely unspecific and coarse, and showed no structural details. Thus, although formation of nuclei takes place at pH 4.5 in higher concentrations of silver nitrate, these nuclei do not lead to specific staining after physical development (fig. 3, G).

(7) Three of the blocking agents which were found to affect silver staining, namely, DNFB, performic acid, and methyl alcohol with hydrochloric acid, were investigated. The sections were pretreated with the reagents as previously described (table 1) and washed thoroughly in several changes of distilled water before impregnation. They were then washed in distilled water for 24 hours

and developed in the physical developer. The effects of these blocking agents on the formation of silver nuclei are shown in table 2.

These experiments show that the silver nuclei are quite stable, and that their formation increases with time, temperature, and the concentration of silver in the impregnating bath. There appears to be an optimum formation of silver nuclei at pH 7-8. When the silver nuclei are formed at low pH values, or if the sections are pretreated with certain blocking agents, the silver nuclei which are formed do not give rise to specific staining on development.

TABLE 2

<i>Blocking agent</i>	<i>Formation of silver nuclei</i>	<i>Amount of reducible silver present</i>
Control (none)	specific	normal
MeOH plus HCl (fig. 3, B)	unspecific	normal
DNFB	unspecific	decreased
Performic acid (fig. 3, D)	unspecific	decreased

(b) *The process of the formation of silver nuclei*

Romanes (1950) considered that the reduction of silver salts in the impregnating stage to produce silver nuclei was brought about either by sulphhydryl groups, ascorbic acid, or aldehydes. The results of blocking the sulphhydryl groups suggests that they do not participate appreciably in the formation of nuclei. To test the effects of ascorbic acid, Romanes immersed sections in solutions of ascorbic acid before impregnation, but he states that although in some cases there appeared to be an improvement in the staining, the results were not conclusive. It is doubtful if ascorbic acid does reduce the silver ions to form nuclei, since the acid is unstable in alkaline solutions though stable in acid solutions. In the present method of staining silver nuclei are not formed to any extent in acid solutions, and even when they are formed they do not lead to specific staining (fig. 3, G). Other evidence has been obtained on this point from the fact that alcohol-fixed sections of scorbutic guinea pig spinal cord give quite normal staining.

If the silver salts in the impregnating bath are reduced chemically, then, as suggested by Romanes, reduction of the silver is probably due to the aldehyde groups. To some extent this appears to be in keeping with the brown colour shown by formol-fixed tissues on removal from the impregnating bath, for sections of tissue from other fixatives do not show the same brown colour after impregnation at 37° C., even though it has been demonstrated that silver nuclei are present. Danielli (1949) and Chu (1950) consider that the aldehyde groups producing the Feulgen's test are those contained in fatty compounds; these are not appreciably affected by formol fixation, which also protects them from later extraction by alcohol. Consequently, these fatty compounds will remain in formol-fixed tissue, and further aldehyde groups might be expected to be derived from the fixative. Most of the aldehyde groups must in fact be derived from the fixative, because sections of frozen tissue

show only a slight reduction of silver on removal from the impregnating solution. On the other hand, alcohol extracts these fatty compounds from fresh tissue (Danielli, 1949), so that the amount of aldehyde remaining in the tissue after alcohol extraction will be small. Such tissue shows no appreciable reduction of silver on removal from the impregnating bath, and can be shown to contain fewer silver nuclei than frozen-dried tissue.

Lhotka and Myhre (1953) showed that treatment of sections with 4 per cent. periodic acid for 2 hours produces an increase in the depth of staining of connective tissue using the method of Foot (1924). They attributed the increase in staining to the freeing of aldehyde groups from 1:2 glycols. Lhotka, Myhre, and Combs (1953) continued these oxidation studies and treated nervous tissue with potassium permanganate, periodic acid, chromic acid, lead tetra-acetate, and sodium bismuthate prior to silver impregnation by a series of different techniques. With two exceptions they found that the oxidation had no effect on the affinity of silver for nerve fibres, and concluded that it is unlikely that the 1:2 glycol radical is present in amounts demonstrable by the procedure used. We pretreated sections of frog sciatic nerve and rat cerebellum with 4 per cent. periodic acid for 2 hours at 25° C. The sections were brown on removal from the impregnating bath, showing that reduction of some silver had been effected, but development produced rather unspecific staining, so that the myelin sheaths of the sciatic nerve and the fibre interspaces were stained. In the rat cerebellum sections staining was also unspecific; in this case few fibres were visible, but staining of capillaries resulted (fig. 3, F). In spite of this different result the general conclusion is the same as that reached by Lhotka, Myhre, and Combs (1953), that the aldehyde groups play no part in the staining of nerves because the release of extra aldehyde groups leads to the staining of connective tissue and not to a strengthening of the nerve staining. As pointed out previously, acidified potassium permanganate reduced the intensity of staining.

Other facts are inconsistent with the idea of reduction of the silver by aldehyde groups. The aldehyde groups were condensed with hydroxylamine by immersing the sections in a solution of 2 gm. $\text{NH}_2\text{OH} \cdot \text{HCl}$, 4 gm. sodium acetate, and 6 ml. distilled water for 80 minutes (Danielli, 1949). After treatment the sections were washed and impregnated. The formation of silver nuclei was found to be unaffected. Similarly, treatment of sections with 0.1 M potassium cyanide before impregnation did not produce any effect.

If the aldehyde groups are responsible for the reduction of silver, then such a reaction could only take place once, because the aldehyde groups would be oxidized to carboxyl groups in the process. It was found that the silver nuclei could be removed from the sections by either 5 per cent. thiosulphate or dilute nitric acid. Sections of alcohol-fixed material were therefore impregnated, treated to remove the nuclei, and then reimpregnated. The reimpregnated sections developed normally, thus indicating the reformation of nuclei. Control slides developed without reimpregnation showed that the nuclei had been removed.

A further factor which will affect the formation of silver nuclei is the stability of the silver solution. It is well known that silver salts are more stable in acid than alkaline solutions, and so it is to be expected that the impregnating solutions will be more unstable at alkaline pH values; consequently an increase in the formation of nuclei might be expected at alkaline pH values. Quantitative information was obtained on this point by taking normal impregnating solutions, made up to have a 1/20,000 concentration of silver nitrate, and a boric acid/borax buffer concentration of 0.01 M. The solution was made up

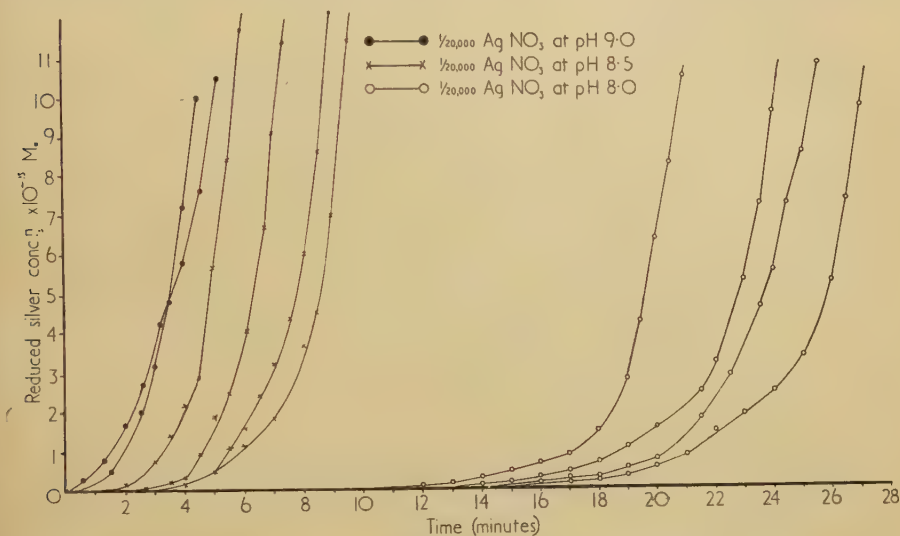


FIG. 1. Reduction of 25 ml. of 1/20,000 silver nitrate, at different pH values, by 3 ml. of 4 per cent. formaldehyde.

fresh before each experiment. As soon as 25 ml. of this solution had been mixed with 3 ml. of 4 per cent. formaldehyde, the resulting solution was transferred to the cell of a 'Spekker' absorptiometer; readings of the silver produced were taken at intervals during the reduction process. To calibrate the 'Spekker' readings, a solution of silver nitrate was completely reduced by formaldehyde, and then diluted to different concentrations. The results of the reduction experiments are shown in fig. 1. These curves are typical autocatalytic curves, and indicate that the number of sites available for the reduction of silver ions in the solution increases with time. This is to be expected, and it is probable that a similar autocatalytic reaction occurs during the process of development when the silver nuclei act as sites of reduction.

Under the conditions of the experiment, reduction of silver began almost immediately at pH 9, and was complete within 5-6 minutes. At pH 8.5 there was a latent period of about 2 minutes, and reduction was complete within 6-9 minutes, while at pH 8 the reduction was much slower, showing a latent period of up to 10 minutes, followed by reduction taking place at different

rates. Reduction was even slower at lower pH values, so that at pH 7.5 there was no indication of reduction in the solution even after 75 minutes. In spite of the small pH range covered by the experiment, there is an appreciable difference in the stability of the different solutions towards reduction. As pointed out by Kubie and Davidson (1928), one reason for this pH effect is that the nitric acid and formic acid produced as a result of the reduction tend to inhibit further reduction. At higher pH values these acids are effectively neutralized.

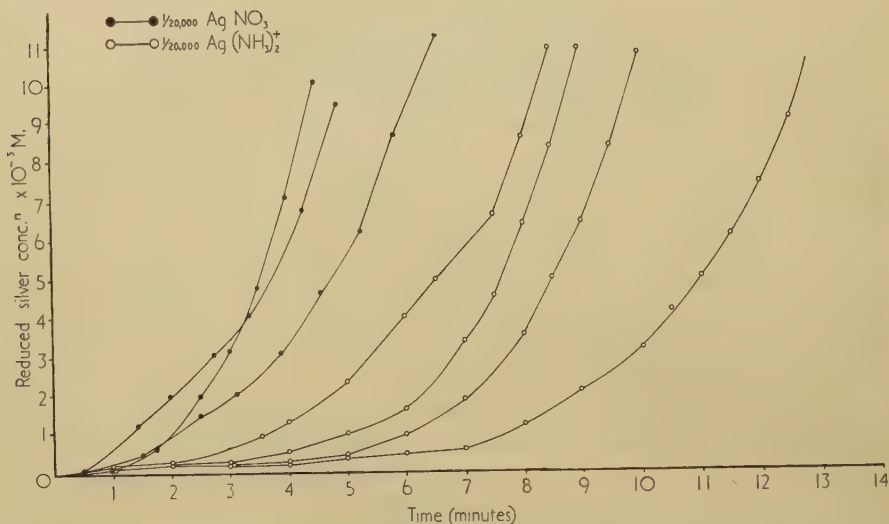


FIG. 2. Reduction of 25 ml. of $1/20,000 AgNO_3$ and $1/20,000 Ag(NH_3)_2^+$, both at pH 9, by 3 ml. of 4 per cent. formaldehyde.

The rates of reduction of the silver ion and the silver diammine ion were compared at pH 9, using equimolar concentrations of Ag^+ and $Ag(NH_3)_2^+$. An initial concentrated solution of $Ag(NH_3)_2^+$ was prepared by the addition of ammonia to a 1 per cent. solution of silver nitrate until the resulting precipitate just dissolved. Again, the solution was made up fresh before each experiment. The results (fig. 2) show that the silver diammine was reduced at about half the rate of the simple silver ion. This is interesting, because there is evidence from experiments, in which the rates of formation of silver nuclei were compared during impregnation in $1/20,000 AgNO_3$ at pH 9 and in $1/20,000 Ag(NH_3)_2^+$ at pH 9, that slightly fewer nuclei are formed in sections impregnated in solutions of the diammine ion.

Romanes (1950) showed that if the pH of the impregnating bath is allowed to fall below pH 7, the resulting stain is granular and unspecific, and photographs produced by Samuel (1953a) show a more granular appearance of the stain at pH 6.8 than at slightly higher pH values. It seems that the silver nuclei formed at lower pH values lead to an unspecific development, because at pH 4.5, although nuclei are formed in more concentrated solutions of silver

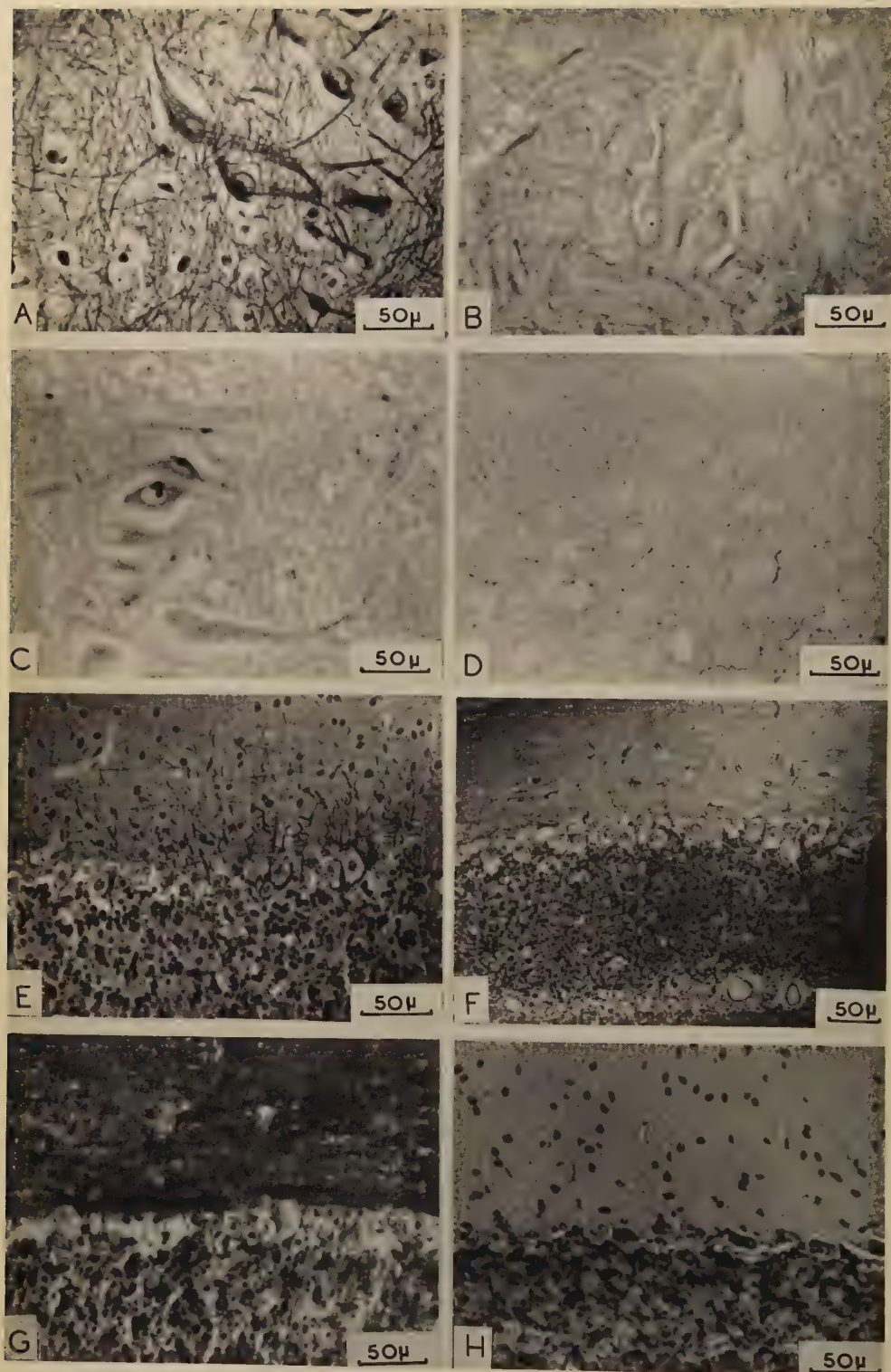


FIG. 3
A. PETERS

nitrate, they do not lead to specific physical development (fig. 3, G). It is probable then, that the nuclei only lead to a specific development when they have been formed at higher pH values (fig. 3, E). However, a similar effect is brought about by the use of a 1/2,000 solution of silver nitrate at pH 9, but in this case there is only a slight loss of specificity due to connective-tissue staining. This is probably brought about by an excessive formation of silver nuclei, with the result that there is some loss of specificity on development.

The process by which nuclei are formed is still in doubt. The results do not distinguish between a physical or chemical reduction of the silver, if in fact such a distinction does exist, because the process may be a combination of both. In the case of formation of nuclei by chemical reduction the aldehyde groups might be responsible, while for physical reduction the silver would be reduced at certain sites by virtue of the redox potential existing there. Factors known to affect the impregnation, such as pH, concentration, and temperature, can support either case. The slow rate of formation of nuclei may be explained either by an initial physical reduction of silver or on the basis that the nuclei have to attain a certain size before they can act as centres for the development of the reducible silver. Similarly, the effect of pH on the reducibility of the silver ions is compatible with either postulate except that there is no explanation for an optimum formation of nuclei at pH 7 to 8. However, whether the initial reduction of the silver is chemical or physical, any further deposition of silver in the impregnating bath on to the existing nuclei is probably due to physical reduction.

The changes brought about by some of the blocking agents which lead to an unspecific development are unknown, but this again points to a physical reduction of the silver in the formation of nuclei, because these blocking agents attack different end-groups in the proteins.

Palmgren (1948) suggests that the power of tissues to reduce silver depends on the fixative employed. Thus he suggests that if the tissue is fixed in indifferent chemical fixatives such as alcohol, then the reducing power of the

FIG. 3 (plate). A, transverse section of alcohol-fixed frog spinal cord showing ventral horn cells. Impregnated in 1/20,000 silver nitrate at pH 9. Developed in hydroquinone-sulphite 10 μ .

B, transverse section of alcohol-fixed frog spinal cord showing ventral horn cells. Esterified before impregnation. Compare with fig. 3, A. 10 μ .

C, transverse section of alcohol-fixed frog spinal cord showing ventral horn cells. Treated with diazomethane before impregnation. Compare with fig. 3, A. 10 μ .

D, transverse section of alcohol-fixed frog spinal cord showing ventral horn cells. Treated with performic acid before impregnation. Compare with fig. 3, A. 10 μ .

E, transverse section of rat cerebellum. Purkinje cell region. Chloral hydrate fixed. Impregnated at pH 9, developed in glycine physical developer. 10 μ .

F, transverse section of rat cerebellum. Purkinje cell region. Alcohol fixed. Pretreated with periodic acid before impregnation at pH 9. Note staining of capillaries and lack of staining of nerve fibres. Compare with fig. 3, E. 10 μ .

G, transverse section of rat cerebellum. Impregnated at pH 4.7. Developed in glycine physical developer. Note lack of differential staining. 10 μ .

H, transverse section of rat cerebellum. Impregnated at pH 9.0. Silver reduced in 2 per cent. hydroquinone. Note lack of differential staining. 10 μ .

tissue proteins is less than if they had been fixed in formol, and further, that many compounds such as chloral hydrate, pyridine, and ammoniated alcohol produce a general increase in the reducing power of the tissues. If Palmgren's hypothesis is correct, then this points to a physical reduction of silver ions to form nuclei in the tissue.

The weight of evidence points to the initial formation of silver nuclei in the tissue by a physical reduction of the silver.

V. GENERAL DISCUSSION

Two processes take place in the impregnating bath—the fairly rapid combination of the free silver ions with the histidine and other amino-acids in the section to form compounded, but unreduced, silver, and the slower formation of silver nuclei. For extensive combination of the silver ions with the histidine it is necessary that the pH value of the impregnating bath should be in the region of pH 9. The effect of pH on the combination of silver ions with histidine has been described by Haarmann and Frühauf-Heilmann (1941), who showed that at pH 7 only 0.41 equivalents of silver are combined with histidine, while at pH 9 the number is increased to 1.12. The process of combination with reduced silver is complete within about 15 minutes at 37° C., but to obtain good staining it has been shown that a longer period of impregnation is necessary because the formation of silver nuclei is a slower process. Hence, although deep staining may be obtained at the end of 2 hours, the details are poor. It is only within the pH range 7.5–9.0 that a balance is obtained between the specific formation of silver nuclei and a sufficient amount of combined silver to produce a deep enough chemical development with developers such as hydroquinone-sulphite.

Willis (1945) believes that combination of silver is at the sites of accumulation of basic proteins, and that these combine with the silver to form complexes. This is in agreement with the hypothesis that it is the histidine which combines chiefly with the silver. Willis does not mention silver nuclei, but does point out that such co-ordination complexes that silver might form would be readily reducible.

The formation of silver nuclei has been assumed since the early paper of Liesegang (1911), and has been confirmed more recently by the work of Holmes (1943), Romanes (1950), and Samuel (1953*a, b*). To a large extent it seems that the factors controlling the combination of unreduced silver with histidine are the same ones that govern the formation of silver nuclei. If the silver is reduced to form nuclei by virtue of the redox potential of the tissues (and this appears to be suggested by the experiments), then it is probably necessary for the silver ions to be combined with the section before they can be reduced to form nuclei.

The position of the silver nuclei is still in question, and their presence is only assumed from indirect evidence. However, the 'latent image' in a photographic emulsion has never been seen, and it is only necessary for relatively

few atoms of silver to be present to form a nucleus for development. If the nuclei form the centres for development, so that the developed silver is deposited on to them, then they must be in the same positions as the developed silver in the finally stained preparation.

Liesegang (1911) thought that the characteristics of the silver nuclei determined the specificity of the stain; thus deeper staining occurred where more nuclei were present. Clearly, to some extent this is true, because without their presence it has been shown that no development can take place. Samuel (1953*b*) has criticized Liesegang's theory because Liesegang failed to remove the reducible silver from the section before development. In his experiments Samuel found that compared with 2½ hours' impregnation, when more nuclei should be present, 15 minutes' impregnation at pH 9.1 resulted in intense axonal staining, although very few silver nuclei should be present. He goes on further to say that one should expect some comparable degree of nuclear deposition after 2½ hours' impregnation at pH 6.8 and 15 minutes' impregnation at pH 9.1, if there is any quantitative correlation between staining and the silver nuclei. To some extent this is true, but there is evidence that the silver nuclei are not formed in the same positions in the section after impregnation at different pH values. Thus it was shown that unspecific staining occurred after the formation of silver nuclei at pH 4.5 (fig. 3, c). It is clear however, that very little is known about the characteristics of the silver nuclei, and it is doubtful if much progress can be made in this direction until the silver nuclei and their positions in the sections can be determined directly.

To summarize, it has been shown that during the impregnating stage a complex series of reactions takes place between the silver ions and the fixed tissue of the sections. Essentially, however, there are two processes taking place, a rapid combination of unreduced silver with the histidine and other amino-acids of the section, and a slower formation of nuclei of reduced silver. To a large extent these two processes are controlled by the same factors of time, pH, temperature, and the concentration of free silver ions in the impregnating bath.

I wish to express sincere thanks to my supervisor, Professor J. E. Harris, for his interest and advice during the course of this work. I am indebted to Professor J. F. Danielli of King's College, London, for advice on the use of protein reagents, and to Dr. J. W. Mitchell of the Physics Department for discussions about the mechanism of photography.

The photographs were taken for me by Mr. K. J. Wood of the Zoology Department.

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Experiments on the Mechanism of Silver Staining

II. Development

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With one plate (fig. 1)

SUMMARY

The effect of a series of photographic developers on the final silver-staining picture has been investigated. Ten common developers were used, but of these only hydroquinone, chloroquinol, pyrogallol, and *p*-aminophenol, were found to be of general use. The other developers were either so weak in their action that the final staining was light and incomplete, or so powerful that a differentiated nerve staining was not produced.

For silver staining to be effected nuclei of reduced silver should be present in the section. These nuclei act as centres for the deposition of additional silver reduced by the developer; the additional silver may either be derived from that combined with the sections during impregnation or from the developing solution itself. Whether or not the additional silver is deposited in such a way as to produce differentiated nerve staining depends on the properties of the developer and on the composition of the developing solution. The redox- and 'bromide'-potentials, the sulphite and hydrogen ion concentrations in the developing solution, and the protective action of the tissue components of the section all play a part in determining the final staining picture.

A new glycine-containing physical developer and a gold thiocyanate physical developer are described.

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I. INTRODUCTION

SO far as I am aware no systematic investigation has been carried out into the use of different developing agents for the reduction of silver taken up during impregnation. The present investigation was undertaken to determine the effect of different developing agents on the final picture obtained in silver staining.

In a previous paper (Peters, 1955) it was shown that during impregnation two essentially different reactions take place between the silver ions in the impregnating bath and the sections of fixed tissue. A substantial part of the

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silver is combined in the unreduced form with the histidine and other amino-acids of the tissue, and a smaller part is reduced to form silver nuclei. The combination of the unreduced silver is a rapid process which is complete within about 15 minutes at 37° C. and pH 9, but the formation of the silver nuclei is a slower process and accounts for the long period of impregnation that is necessary to obtain good staining. During development the silver nuclei act as centres on which the developing agent can deposit additional silver derived from the combined (reducible) silver fraction.

Liesegang (1911) considered that the characteristics and distribution of the silver nuclei determined the final specificity of the stain. To some extent this is true because they act as the centres for the reduction and deposition of the reducible silver. However, the characteristics of the developing solution also play a part in the final distribution of developed silver. Therefore the final staining picture depends both on the distribution of the silver nuclei and on the action of the developing agent in depositing additional reduced silver in relation to these nuclei.

II. DEVELOPMENT

In photography developers are classified into two main types, namely, 'physical' and 'chemical' developers (Mees, 1944). In physical development the silver which is deposited onto the latent image centres of the exposed emulsion is derived from the developing solution, so that the emulsion need contain no silver other than that of the nuclei which constitute the latent image. Such a developing solution contains a reducing agent, free silver ions, and a protecting or complexing agent to retard the action of the developer on the free silver. In contrast, in chemical development, the silver reduced to form the visible image is that of the silver halide crystals in the emulsion. Therefore a chemical developing solution contains no free silver ions initially. In general the developing agents used in chemical development are stronger than the ones used in physical development. The developing agents most widely used in silver staining are hydroquinone and formol.

In the case of an impregnated section, development is always physical in nature since free silver ions are involved; there is no mechanism like the chemical development of an emulsion, which involves crystals of silver halide containing nuclei of reduced silver. There are, however, two possible sources of silver ions which can be reduced by the developer. The silver ions may be derived either from the reducible silver combined with the tissue during the impregnating stage (see Peters, 1955), or from the developing solution. If the reducible silver is not removed from the section before it is immersed in a developing solution, itself containing free ions, then the developed silver will be derived from both the section and the solution.

For the sake of clarity, in the present discussion, although there is no parallel with chemical reduction in the photographic sense, developing solutions containing free silver ions initially will be referred to as 'physical developing

solutions', and the developing solutions containing no free silver ions initially as 'chemical developing solutions'.

(a) *Physical developing solutions*

Pearson and O'Neill (1946), suggested the use of a physical developer containing hydroquinone with gelatine as a protecting agent. The developer that they used had the following formula:

1 per cent. hydroquinone .	2 ml.	} at 60° C.
2 per cent. AgNO ₃ .	5 ml.	
3 per cent. gelatine .	20 ml.	

The pH value of the solution was adjusted to 4.4 by citric acid. This developing solution was tested but was found to produce rather a granular development which was only specific when the sections had been impregnated at pH 7 or 8. Hydroquinone is probably too strong a reducing agent to be used in this type of development, and consequently other developing agents were tried. The best results were obtained with a developer having the composition:

Stock solution: glycine	1.25 gm.	} 20 ml.
Na ₂ SO ₃ (anhyd)	2.5 gm.	
5 per cent. gelatine	25 ml.	
distilled water	225 ml.	
0.1 M citric acid-sodium citrate buffer at pH 6.3	20 ml.	
1 per cent. silver nitrate solution	1 ml.	

The mixing of the stock solution with the silver nitrate and buffer is carried out just before the solution is to be used because the mixture is unstable: silver begins to plate out from the solution within 10–15 minutes of mixing. The usual time for development is of the order of 5 minutes or even less. Slides should be rinsed in distilled water before immersion in the solution (fig. 1, H).

In general the optimum pH value for development was pH 6.3, but in some cases a more selective staining was obtained at pH 6.0. However, the isoelectric point of the gelatine is important in such a solution, and for the sample used here it was at pH 5.3. To obtain the best results tests should be carried out over the range pH 5.5–6.5 since on either side of the optimum the deposition of silver is granular. This is especially noticeable on the acid side. The citric acid-sodium citrate buffer is used to control the pH value at the points of reaction, since a change of pH at the deposition sites during development may influence the further deposition of silver.

Two other means of improving the results may be used:

- (i) The development is carried out at low temperatures, which retards the deposition, so that finer grains of silver are produced, or
- (ii) The volume of silver nitrate solution in the formula is reduced to 0.5 ml. This has virtually the same effect as (i).

Other developing agents were added to the above type of solution. With hydroquinone, metol, and chloroquinol the staining was unspecific and the silver deposition was granular.

(b) *Chemical developing solutions*

Ten common photographic developers were used; hydroquinone, chloroquinol, metol, glycine, pyrogallol, pyrocatechin, amidol (2:4 diaminophenol), phenylenediamine, *p*-aminophenol (base), and oxalic acid. Most of the tests were carried out on alcohol-fixed sections of frog spinal cord, but many experiments were repeated on alcohol-fixed rat cerebellum and formol-fixed human cerebrum and cerebellum. In many cases the sections were afterwards toned with gold. The developing solutions also contained sodium sulphite, and in a few tests citric acid was added. The only developers which were found to be of general use were hydroquinone, chloroquinol, *p*-aminophenol, and pyrogallol; others gave unspecific or faint results, so that the details of the nervous system were not clear.

The amidol solution used had the composition:

amidol	0.5 gm.
Na ₂ SO ₃ (anhydrous)	5 gm.
distilled water	100 ml.

The pH value of the solution was varied by the addition of 10 per cent. caustic soda or 25 per cent. acetic acid, and determined just before use by a pH meter. This solution gave the best results at pH 6.5, which agrees with the value obtained by Davenport, Bruesch, and McArthur (1939), who used a similar solution after impregnation in protargol. (The developer used by them was similar in composition to the one used here, but with the addition of 5 gm. of crystalline sodium sulphite.) At other pH values the silver deposit is granular. The developer is poor for normal routine staining, since there is a tendency for the connective tissues and myelin sheaths to stain. This lack of specificity was also found by Samuel (1953*a*), who used a 1 per cent. amidol-

FIG. 1 (plate). A, transverse section of rat cerebellum. Purkinje cell region. Alcohol fixed. Impregnated at pH 9.0. developed in metol-sulphite. 10 μ .

B, transverse section of rat cerebellum. Purkinje cell region. Alcohol fixed. Impregnated at pH 9.0. Developed in glycine-sulphite. 10 μ .

C, transverse section of rat spinal cord. Alcohol fixed. Impregnated at pH 9.0. Developed in *p*-aminophenol-sulphite. 10 μ .

D, section of human cerebellum. Formol fixed. Impregnated at pH 9.0. Developed in pyrogallol-sulphite. 10 μ .

E, transverse section of rat cerebellum. Purkinje cell region. Alcohol fixed. Impregnated at pH 9.0. Developed in hydroquinone-sulphite. 10 μ .

F, transverse section through brain stem of rat; cerebellar region. Alcohol fixed. Impregnated at pH 9.0. Developed in chloroquinol-sulphite. 10 μ .

G, transverse section through brain stem of rat; cerebellar region. Alcohol fixed. Impregnated at pH 9.0. Gold thiocyanate development. 10 μ .

H, transverse section of rat cerebellum. Purkinje cell region. Alcohol fixed. Impregnated at pH 8.0. Developed in glycine physical developer. 10 μ .

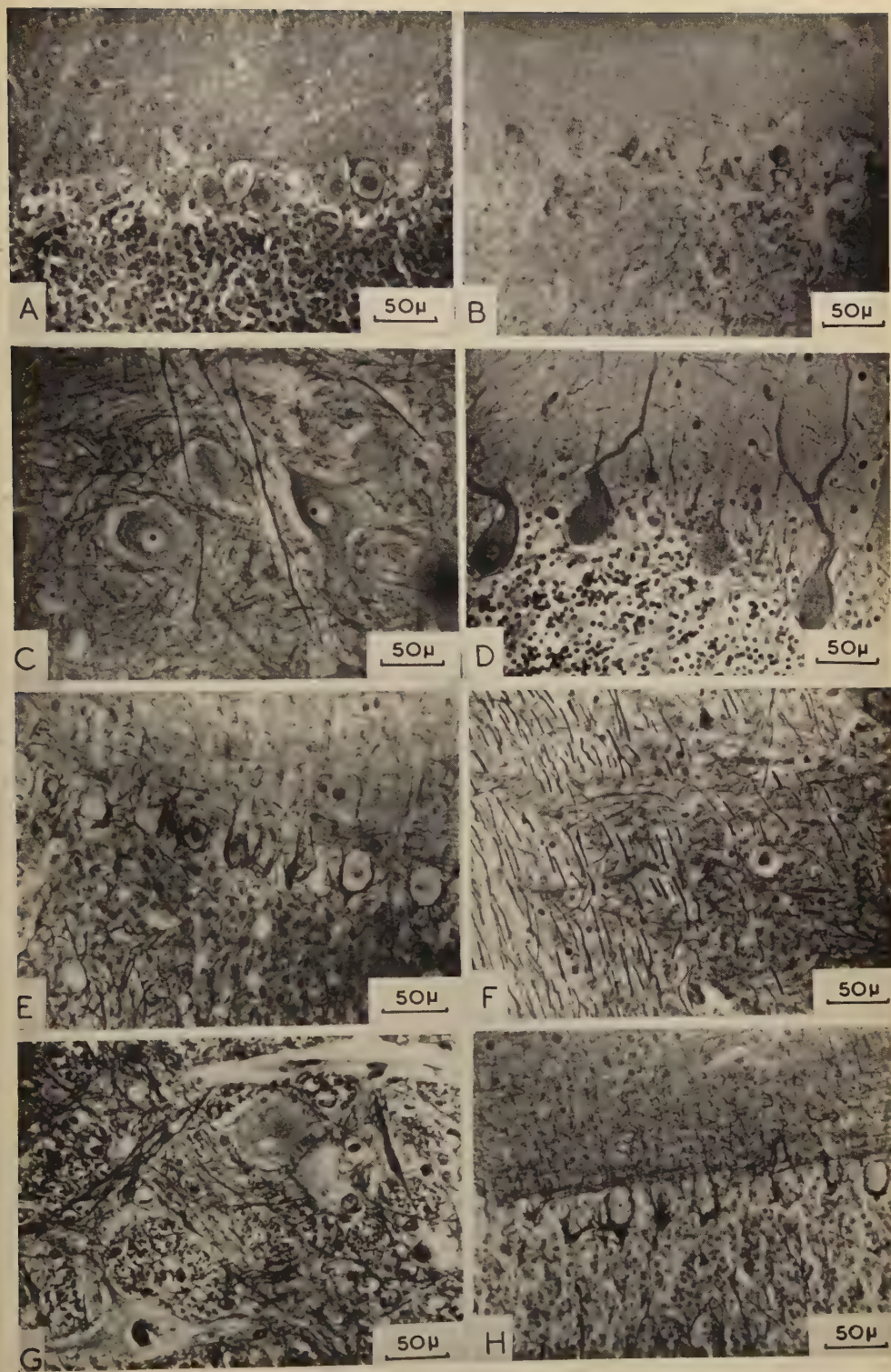


FIG. 1
A. PETERS

sulphite developer. It is, nevertheless, a useful developer for fine connexions and cell bodies.

Metol, at a concentration of 0.5 per cent. or 0.25 per cent. with the addition of sulphite, gave a very unspecific staining picture (fig. 1, A). The addition of citric acid to the solution produced very little improvement. In the presence of citric acid alone the development was weak.

Para-phenylenediamine, as in the case of metol, gave an unspecific and granular deposition of silver, which resulted in an extensive staining of connective tissue.

Formol, used as a 10 per cent. solution (4 per cent. formaldehyde), was very active and produced a deep, but unspecific staining. This is not surprising because formol is commonly used for development in silver methods employing frozen sections.

Glycine, either in the presence of sulphite or with the addition of citric acid, produced very light development in which few details were visible (fig. 1, B). This is also true of oxalic acid and pyrocatechol.

Good results were obtained with the remaining four developing agents. The *p*-aminophenol was used in the form of the base, which is not soluble in water, and consequently the developer was initially dissolved in absolute alcohol. The composition of the solution employed was:

<i>p</i> -aminophenol (dissolved in 40 ml. abs. alcohol)	. 0.5 gm.
Na ₂ SO ₃ (anhyd)	. 5.0 gm.
distilled water	. 60 ml.

The solution gave good staining, with a small amount of connective tissue staining, which did not obscure the details of the fibres appreciably (fig. 1, c). If the concentration of the sulphite was reduced to 2 per cent. or the *p*-aminophenol was used in a completely aqueous solution (when caustic soda had to be added to dissolve the developer), the results were not so good.

The developing solution containing pyrogallol had the composition:

pyrogallol	. 2 gm.
Na ₂ SO ₃ (anhyd)	. 2 gm.
distilled water	. 100 ml.

This solution produced deep development, with quite extensive nuclear and fibre staining (fig. 1, d). The addition of extra sulphite to the solution, resulted in a less intense staining.

Chloroquinol produced results very like those obtained after development in warm solution of hydroquinone. This might be expected, since the two compounds have a similar structure. The formula of the developing solution used was:

chloroquinol	. 1 gm.
Na ₂ SO ₃ (anhyd)	. 4 gm.
distilled water	. 100 ml.

To make up this solution, the whole was warmed to dissolve the chloroquinol, cooled, and then filtered. Development took 4 minutes at room

temperature. The results were more complete than those produced by hydroquinone at room temperature, but very similar to those obtained when the hydroquinone developer was warmed to 20° C. (fig. 1, F).

The hydroquinone solution had a similar composition to that employed by Holmes (1943) and Romanes (1950), namely:

hydroquinone	. 1 gm.
Na ₂ SO ₃ (anhyd)	10 gm.
distilled water	. 100 ml.

This contains twice the amount of sulphite used by Holmes and Romanes, but the addition of extra sulphite resulted in a more differentiated staining (fig. 1, E). The effect of sulphite in increasing the differentiation of the stain was described by Davenport and Kline (1938). If the concentration of the sulphite was less than 5 per cent. then the staining was granular and less specific. It can be shown that the depth of staining on development decreases with the addition of sulphite to the solution and, conversely, increases with the addition of more hydroquinone. Temperature is also an important factor, and Romanes (1950) put the optimum temperature at 15° C. Experiments showed that the intensity and extent of staining increased with temperature. At 0° C. the staining was very light and few cell nuclei were stained, but as the temperature was raised, the numbers of nuclei stained, together with the details of nerve fibres, increased. It is therefore suggested that the developer should be warmed to a temperature of about 20° C. before use. Samuel (1953*a*), showed that at low pH values the activity of hydroquinone developers is reduced.

Reinders (1934) produced a series of ferrous citrate-ferric citrate developing solutions. These solutions consisted of 20 ml. of a solution containing 0.125 M ferrous ammonium sulphate and 0.125 M ferric ammonium sulphate, to which was added different volumes of 1 M sodium citrate to vary the redox potential. A redox potential of +0.06 volts was obtained by the addition of 10 ml. of citrate, while the potential of the solution without the citrate was +0.645 volts. Hence, by varying the amount of citrate added the redox potential of the solution could be varied and adjusted to intermediate values.

To find the effect of redox potential on the development of the silver-impregnated sections, frog central nerve cord sections were developed in solutions of different potentials. There was only a slight development above +0.45 volts, but immediately below this value a rapid rise in the intensity of staining occurred, followed by a gradual rise in intensity as the potential fell from +0.45 volts to 0.06 volts. The staining produced by this developer was too light to be of general use, but this experiment showed the importance of the redox potential of the developer in staining.

The varying types of staining produced by different developers suggested that there was some movement of the reducible silver during development. This was shown by the following experiment. Three slides were impregnated in the same solution. The first was developed in hydroquinone-sulphite, the

second in amidol-sulphite, and the third in hydroquinone-sulphite for 10 seconds followed by amidol-sulphite. Specific staining was obtained in the first and last slides, both having approximately the same intensity of staining. In the slide developed in amidol alone, the staining was more intense and less specific than in the other two. One must therefore conclude that in the third slide, during the short immersion in hydroquinone and before it was transferred to the amidol, silver was lost from the section. Otherwise the amidol would have produced some connective tissue staining and the staining would have been as deep as that obtained in the second slide where the amidol alone was used. A similar experiment was carried out by Samuel (1953*a*), but he immersed his sections in hydroquinone for 5 minutes before transferring them to the amidol solution. His experiment does not show the rapidity of the loss of reducible silver from the section when it is immersed in the developing solution.

(c) Discussion

From the above experiments on development it is seen that of the chemical developers considered only four are useful. Once the silver nuclei have been formed in the section the staining depends to a great extent on the developer although the tissue of the sections also plays an important part. Thus, even among the four good developing agents, pyrogallol produced poor staining in the rat cerebellum sections although it produced good results in the other tissues which were tested.

The effect of the potential of the developer on the reduction of the silver has been shown to be important; to some extent the development is governed by the equation:

$$E = E_{Ag} - E_{redox}$$

where E_{Ag} is the silver potential,
 E_{redox} , the potential of the developer, and
 E , the resulting potential.

For the silver ions to be reduced, it is necessary that E should reach a minimum value for any given system (Reinders, 1934). By variation of E_{redox} , the minimum value for this factor can be determined, and for the ferrous-ferric citrate system the value has been shown to be +0.45 volts. In practice, however, the value of the redox potential is of little help in determining the type or rate of development that will be produced by a series of different developing solutions, since it is merely an index of the ratio of reduced to oxidized form of the developing agent. The actual rate of development depends upon the activity of the developer at the site of action; this is determined by such factors as the rate of diffusion of the developer.

Bromide retards the action of a developer, and the amount of bromide that it is necessary to add to a series of developers to reduce their activity to a given level provides a characteristic index for each developer. This is called the 'bromide potential' of the developer (Mees, 1944, p. 352). The bromide potentials

of some of the developing agents used in the experiments are shown in table 1.

In general the developing agents which produce specific development are those with low bromide potentials, while the ones that produce unspecific development have higher bromide potentials. One exception to this is *p*-phenylenediamine, which has a low bromide potential and yet produced unspecific staining.

The various types of development brought about by the different developing solutions brings out another important point. If the silver nuclei are confined to the nervous elements then the staining should be specific irrespective

TABLE I

The bromide potentials of developers

<i>Developing agent</i>	<i>Bromide potential</i>
Ferrous oxalate . . .	0.3
<i>p</i> -phenylenediamine . . .	0.3
Hydroquinone . . .	1.0
Glycine . . .	1.6
<i>p</i> -aminophenol . . .	6.0
Chloroquinol . . .	6.0-7.0
Pyrogallol . . .	16.0
Metol . . .	20.0
Amidol . . .	30-40

of the developing agent used. The fact that strong developing agents, such as metol, produce unspecific staining, suggests that all elements in the section contain silver nuclei. With a weak developing agent the nerve fibres stain, but when stronger developing agents are used the cell-nuclei, cell-body, and connective tissue stain. Which silver nuclei in the section act as centres for development depends on a series of factors, of which the most important are the size of the silver nuclei, the numbers of the silver nuclei, the activity of the developing solution, the distribution of reducible silver, and the protective action of the protein at the site.

The protective action of protoplasm in staining has been discussed at length by Zon (1936). The protective action of a system may be regarded as the influence which that system exerts in retarding a chemical reaction, in this case the reduction of silver by the developing agent. With any given developing solution the elements which stain most readily are the ones with the lowest protective action and the largest silver nuclei. When strong developing agents are used the protective action of the elements containing the nuclei is overcome, so that elements in the section stain in the sequence outlined above. Specific staining is therefore the result of the deposition of developed silver on the nuclei contained only in certain tissue elements.

Variations in development may be brought about by the action of sulphite in the solution. Sulphite can change the potential of the developing solution

quite appreciably (Evans and Hanson, 1937). The action of sulphite is complicated, but it is known to form complexes with silver and to increase the rate of development by rapidly removing the oxidized developer in the form of sulphonate from the site of development (Evans and Hanson, 1937). Samuel (1953a) has shown that sulphite competes with the developer for the reducible silver held by the section. He found that immersion of an impregnated section in a 2.5 per cent. solution of sodium sulphite for 2 minutes before development removed the silver which would have been reduced and deposited by the developer. Therefore no visible staining occurs at the development stage if the developing solution does not contain free ions of silver. Samuel concluded that hydroquinone, which is a relatively weak developing agent, was partially outpaced by the sulphite, which removed a greater portion of the reducible silver from the section than when amidol was used. Thus amidol, a more powerful developer than hydroquinone, outpaced the sulphite and reduced the silver *in situ*. Samuel also established that low pH values facilitated the removal of silver by the sulphite. This is to be expected, because lowering the pH value reverses the ionization of the basic groups of the proteins and so releases the silver ions from combination.

Holmes (1943) stated that the pH of the developing solution is not critical in determining the specificity of staining. To some extent this is true in chemical development since, within limits, the pH value of some solutions can be changed by the addition of citric acid without producing any great effect, but the experiments with amidol show that the pH can affect the rate and type of development. In physical development, on the other hand, pH is a much more critical factor, and is only variable within small limits.

Thus the final staining picture is dependent on the activity of the developing agent, the concentration of sulphite, and hydrogen ions in the solution, and the protective properties of the elements of the section at the site of development. Whether the silver reduced by the developer is derived from the section or the developing solution, no staining can be obtained unless the section has been previously impregnated, so that silver nuclei have been formed in it.

III. GOLD THIOCYANATE DEVELOPMENT

James, Vanselow, and Quirk (1948) showed that treatment of exposed photographic emulsions with an aurous thiocyanate solution increased the rate of subsequent development. The silver of the latent image appeared to be either replaced, or plated over, by gold. However, the action of gold did not end with a replacement because if the exposed emulsion was allowed to remain in the aurous thiocyanate solution, then a visible image appeared, thus showing that physical development of the latent image, by the gold, was taking place.

James (1948) later showed that prolonged treatment of exposed emulsions by aurous thiocyanate, produced gold particles which were visible under the electron microscope. James considered that the deposition was autocatalytic,

and that the effect of the gold was to build up the latent image nuclei far above their normal size.

To obtain further evidence of the formation of silver nuclei during the impregnation of nervous tissue and to investigate aurous thiocyanate as a possible developing agent, sections pretreated with 1/20,000 silver nitrate, at pH 9 and 37° C. for 20 hours, were immersed in an aurous thiocyanate solution. The basic solution of aurous thiocyanate was made as follows: 40 ml. of a 0.1 per cent. solution of KAuCl_4 was heated to boiling, when 0.6 gm. of potassium bromide and 0.5 gm. of potassium thiocyanate were added. When the solution of the latter was complete, the whole was cooled and diluted to 160 ml.

After impregnation in the silver nitrate solution the sections were washed in several changes of distilled water for 30 minutes to 1 hour to remove uncombined silver ions, and then immersed in the following solution at 37° C.:

25 ml of solution of AuCNS complex
5 ml. of 0.5 per cent. gelatine
20 ml. of distilled water.

The period of immersion in the gold complex was generally of the order of 24 hours at 37° C., but the sections can be removed when a sufficient colour depth is obtained. The aurous thiocyanate is rather unstable and tends to reduce to gold easily. As in the case of the physical developer containing silver, the gelatine acts as a protective colloid to retard the deposition of gold from the solution.

The results were generally good and the staining specific in almost every case (fig. 1, G); the staining was rather like that obtained after gold toning of silver stained sections.

Experiments were carried out with the thiocyanate solution in an attempt to obtain staining without pretreatment with silver nitrate. In these experiments the pH, concentration, temperature, and absence of potassium bromide were tested, but the results were much inferior to those obtained after pretreatment with silver nitrate or after normal silver staining.

The production of specific development by the gold thiocyanate after pretreatment with silver nitrate is further evidence for the formation of silver nuclei during the period of impregnation in the silver solution.

The outstanding features of the staining produced by this method of development were the deep staining of the nerve-cell nuclei and the extensive staining of the cytoplasm of the cell-bodies (fig. 1, G).

IV. GENERAL DISCUSSION

In photography the 'latent image' determines the sites of development, and it is believed to consist of sensitivity specks of reduced silver which, on development, allow the silver bromide crystals containing them to be preferentially reduced by the developer. This being the case there is a parallel between the sensitivity specks of the emulsion and the silver nuclei in the sections.

The mechanism of development in photography is far from understood (James, 1950). The fundamentals are known, however, and in simple form a corresponding sequence taking place during chemical development of the impregnated section may be outlined as follows. On immersion of the section in the developing solution an unstable system results such that the reducible silver combined during the impregnation stage tends to diffuse away from the section and to form complexes with the developing agent or any other complexing agents such as sulphite, which may be present in the solution. The rapidity of the reaction is seen from the experiment in which a section was transferred to amidol after development for 30 seconds in hydroquinone. Initially, therefore, there will be a high concentration of complexed silver in the interstices of the section. When a strong developing agent such as metol is used there will be little diffusion of silver away from the section, and it will be reduced *in situ*, so that an unspecific staining results (fig. 1, A). With other developing agents, such as hydroquinone and chloroquinol, the silver will be less readily reduced and will be deposited at specific points so that differential staining occurs in relation to the position and number of silver nuclei present (fig. 1, E and F). Taking this even further, weak developing agents which reduce even less rapidly deposit only a small portion of the reducible silver, the rest being lost to the solution, so that the staining is only light (fig. 1, B). The deposition of silver on to the nuclei is autocatalytic. It is probable that during differential development, only certain silver nuclei act as centres for the deposition of silver. On the other hand, in development with stronger developing agents, the silver may be deposited on a more extensive range of nuclei, so that an unspecific staining results. This of course implies a differential deposition of the developed silver in relation to different silver nuclei.

In physical development it is only necessary for the silver nuclei to be present in the section for development to be possible. Thus the silver, which is complexed with the tissue elements and reduced during chemical development, may be removed from the section either by washing or by the action of sodium sulphite (Samuel, 1953b). If the reducible silver is not removed from the section before physical development then it will be available to the developer.

During development it is believed that an activated complex is formed between the silver and the developer (James, 1950). In the case of hydroquinone it is thought that the complex has the form, hydroquinone:Ag⁺:Ag metal, which splits up into oxidized developer and reduced silver. This type of reaction is probably common to a large number of developers, although in those like *p*-phenylenediamine the absorption of the developer by the silver nuclei is important. Whether the silver ions are derived from the section or from the developing solution as in physical development, the reaction is virtually the same.

A further important factor is the 'protective action' of the tissue of the section. This has been discussed at length by Zon (1936) who found that when silk fibres were immersed in a solution of silver nitrate and potassium dichromate

in gelatine, the fibres took on the distinct red colour of silver dichromate. A precipitate was not formed in the gelatine solution for several hours, showing that the protective action of the silk fibres was less than that of the gelatine. Thus the protective action of the gelatine may perhaps be likened to that of the connective tissue, and that of the silk fibres to the nerve fibres. Consequently, the different protective properties of the tissue elements may play an important part in the deposition of silver, both during impregnation and development. In development using strong developing agents, the protective action of the tissues is not so important, so that an unspecific staining results, but with weaker developing agents it is more important and produces a more specific deposition of developed silver in relation to the silver nuclei.

Before any development can take place it is necessary that silver nuclei should be formed in the section. These are formed during the impregnating stage (Peters, 1955), and the reactions which take place between the sections and the silver ions in the impregnating bath can exert a considerable influence on the final staining picture. Thus Silver (1942) has shown that the pH value at which impregnation is carried out determines which elements in the section stain on development.

Silver (1942), in his paper on the colloidal factors controlling silver staining, states that the absorption of negatively charged micelles of silver by the regions of the tissue bearing positive charges is the factor controlling the specific deposition of silver during staining. The negatively charged micelles are assumed to be formed by the action of the developer on the reducible silver, so that specific deposition does not take place until the sections are immersed in the developer. Samuel (1953*b*) has shown that Silver's hypothesis is incorrect. Although the developing agent plays some part in the specificity of staining, as pointed out by Holmes (1943), Silver makes no mention of the presence of silver nuclei, and assumes that specific deposition is determined solely by the charges on proteins. While the charge on the proteins may determine the sites of formation of the nuclei, it is principally the nuclei that determine the specific deposition sites.

Palmgren (1948) has given a theoretical treatment of the mechanism of silver staining, and the present theory is largely in agreement with the theory put forward by him. He stresses the importance of the formation of silver nuclei, and states that the nuclei have a negative charge, so that they absorb the positively charged silver ions. However, the initial charge on the nuclei is unknown, but it is probable that on immersion in the developing solution they assume a negative charge. Palmgren also points out that a weak developing agent is necessary to ensure specificity of staining, so that the silver, as yet unreduced, has time to move out of the tissue between the nervous elements. To slow down the rate of development by pyrogallol he added alcohol to the developing solution.

The action of the developer is therefore seen to be complex. Development can be affected, and to some extent controlled, by a number of factors, so that the deposition of developed silver is varied in relation to the silver nuclei.

Thus it is clear that in a series of sections impregnated under identical conditions the type of final staining picture obtained depends on the developing solution which is used to reduce the developable silver.

I wish to express sincere thanks to my supervisor, Professor J. E. Harris, for his interest and advice during the course of this work. I am indebted to Dr. J. W. Mitchell of the Physics Department for discussions about the mechanism of photography and the use of developers.

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The Significance of the Presence of Pollen in the Food of Worker Larvae of the Honey-bee

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SUMMARY

1. From a study of the quantity of undigested shells of pollen grains in the gut of worker honey-bee larvae it is concluded that the larvae receive a variable quantity of pollen in their food in summer and sometimes none at all in winter.

2. Probably less than one-tenth of the nitrogen requirements of growing larvae are obtained from the pollen in their food.

INTRODUCTION

WORKER larvae of the honey-bee are fed on a fluid which is, at least in part, a secretion of the pharyngeal glands of the adult worker bees (Schiemenz, 1883; Langer, 1912). Köhler (1922) and others have found many pollen grains in the food of larvae over 3 days old, but few in the food of younger larvae. Since it was not known what contribution this pollen makes to larval nutrition, estimates have been made of the quantity of pollen swallowed and the amount of nitrogen which it can provide.

METHODS

Pollen grains in the larval gut

The gut contents of fully grown worker larvae were stained and suspended in 1 c.c. of glycerine and an estimate by haemocytometer counts was made of the number of shells of pollen grains in each gut. These estimates represented the total amount of pollen swallowed by the larvae, since the mid-gut and Malpighian tubules have no connexion with the hind-gut in the larval stage of the honey-bee (Snodgrass, 1925) and the shells of the pollen grains are not digested (Parker, 1926; Whitcomb and Wilson, 1929). The estimate of the mean number of pollen grains per larva for each group is given in table 1.

Comparison of the variation between 10 counts on separate larvae with that between 10 counts on one larva shows that there were differences in the number of pollen grains in individual larvae ($P < 0.001$). The larvae examined on 23 February 1953 contained nearly as many pollen grains as those examined in summer, but there were fewer grains in the larvae examined on 10 February. No pollen was found in the larvae from the first comb of colony *A* (although the gut of one larva was stained and crushed under a coverslip and examined as a whole); this comb contained no stored pollen. The other larvae came from combs in which pollen in good condition was stored within the area covered by bees; all the pollen grains identified in winter larvae were from summer flowering plants and could not have been recently collected.

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Pollen available in the field

Samples of trapped pollen, each of approximately 5 g. (i.e. about 1,000 pellets of pollen) from a 1 to 3 days' catch were moistened, thoroughly mixed, and dried in an incubator at 33° C. From each sample duplicate portions

TABLE 1

Estimated number of pollen grains in the gut of fully grown worker larvae

<i>Date of sample</i>	<i>No. of larvae</i>	<i>Mean no. of pollen grains (thousands)</i>	<i>S.E. of mean (thousands)</i>
c. 25 May 1952 . . .	17	162	16
17 and 18 July 1952 . . .	6	138	28
8 August 1952 . . .	10	188	27
8 August 1952 . . .	1	122	8
	(10 counts)		
10 February 1953			
Colony A, 1st comb . . .	5	0	0
Colony A, 2nd comb . . .	2	34	28
Colony B . . .	7	27	3
23 February 1953 . . .	10	122	24
20 August 1953 . . .	6	222	32

(about 30 mg.) were taken for nitrogen determinations by the micro-Kjeldahl method and about 100 mg. of the remainder was stained and suspended in 25 c.c. of glycerine. Ten fields of the haemocytometer were counted for each sample. The analysis of these samples (table 2) shows a mean nitrogen content

TABLE 2

Number of grains per mg. and per cent. nitrogen in samples of trapped pollen

<i>Date of sample</i>	<i>Pollen grains per mg. pollen (thousands)</i>	<i>Per cent. N</i>
9 June 1951 . . .	92	4.35
30 June 1952 . . .	126	3.52
6 July 1952 . . .	108	3.76
24 July 1952 . . .	65	2.57
13 August 1952* . . .	22	3.52
29-31 August 1952† . . .	29	3.86
MEAN . . .	74	3.59

* 99 per cent. red clover pollen. † 88 per cent. red clover pollen.

of 3.59 per cent. of the dry weight, which is close to that found by Todd and Bretherick (1942). It was observed that 99 per cent. (count of 1,000 grains) of the pollen in the sample of 13 August 1952 came from red clover (*Trifolium pratense*).

The nitrogen content of the pollen swallowed by larvae

(a) *Summer.* Combination of the data in tables 1 and 2 gives a mean of

0.078 mg. pollen nitrogen per larva for the summer larvae of 1952 but this estimate is probably very inaccurate because of the variation in size of the pollen grains of different plant species. The error can be considerably reduced for the larvae examined in August 1953 which contained only pollen from red clover, white clover, and sainfoin. For each of these larvae the proportion of pollen of each species was estimated (count of 1,000 grains) and after correction for grain size the amount of nitrogen represented by the pollen was calculated from the analysis of the nearly pure sample of red clover pollen obtained on 13 August 1952. This method gave a mean value of 0.19 mg. N per larva (5 per cent. fiducial limits 0.14 to 0.23 mg.).

(b) *Winter*. The larvae from colony *B* on 10 February 1953 contained only red clover pollen and gave a mean of 0.042 mg. N per larva (5 per cent. fiducial limits 0.032 to 0.053 mg.).

DISCUSSION

It has been estimated that 145 mg. (Alfonsus, 1933) or 125 mg. (Rosov, 1944) of pollen are required to rear one worker larva; Haydak (1943) calculated that $3\frac{1}{2}$ mg. of nitrogen were required. Melampy and others (1940) found that the bodies of mature worker larvae contained about 2 mg. of nitrogen but this quantity probably does not include the whole of the nitrogen in the pollen which the nurse bees used in producing pharyngeal gland secretion.

The contents of the pollen grains found in larvae in the present observations had been digested and so had presumably contributed to the nutrition of the larvae, but this pollen had probably not supplied more than a tenth of the nitrogen requirement of the summer larvae, while some of the winter larvae received much less pollen or none at all. The quantity of nitrogen supplied by pollen is insufficient even to supply the amount needed after the first 3 days of larval life (during which little pollen is present in the larval food), since Melampy and others (1940) found that larvae 3 days old contained only about 0.25 mg. of nitrogen. Haydak (1937) found that colonies without access to pollen continued to rear larvae for some time, the larval food being derived from reserves in the bodies of the adult bees. The young bees produced in Haydak's experiment might have been deficient in some respect, but no deficiency is likely to be associated with normal winter brood rearing, which occurs at a time when the adult bees have a considerable accumulation of protein in their fat bodies (Lotmar, 1939; Maurizio, 1950). Pollen is therefore not an essential constituent of the food of worker larvae, and the scarcity of pollen usually observed in the food of queen larvae cannot account for their differentiation from worker larvae.

Feeding on pharyngeal gland secretion makes possible the very rapid growth of young larvae and simplifies digestion and faecal disposal; hence it is difficult to understand why the larvae should receive any pollen directly. Haydak's (1943) suggestion that the presence of pollen in larval food is due to contamination of the mouth-parts of the nurse bees while eating pollen seems inadequate to explain the quantity found in larvae. Since, however, the

sugar content of the food given to larvae of different ages appears to vary (v. Planta, 1888, 1889; Haydak, 1943) a more probable explanation is that the food receives an addition of sugary material from the bee's honey stomach which, in a nurse bee, will frequently contain some pollen in the process of being transferred to the ventriculus by the mechanism demonstrated by Bailey (1952).

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Neoteny with Goitre in *Triturus helveticus*

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With 2 plates (figs. 1 and 2)

SUMMARY

A population of newts from a pond near Crail, Fife, was found to contain neotenic and goitrous specimens of *Triturus helveticus* in 1951 and 1952 but not in 1953 and 1954. This is a unique record of goitre among amphibians.

The thyroid glands of normal adult and neotenic non-goitrous *T. helveticus* are paired spheroidal or ellipsoidal bodies whose longest dimension varies between 300 and 700 μ . They consist of a few large follicles. The thyroid glands of the goitrous neotenic specimens may be as much as 4 mm. long. They are extremely hyperplastic and hyperaemic and for the most part consist of follicles smaller than those characteristic of normal newt thyroids. These goitrous thyroids produce major displacement of other structures in the throat region but do not invade other tissues.

It is suggested that the goitres result from the exposure of overwintering newt larvae to the 'brassica factor' carried to the pond in the faeces of rabbits feeding on turnips and kale in neighbouring fields.

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INTRODUCTION

THE phenomenon of neoteny is well known in urodeles, but, so far as we are aware, none of the many records in the literature mentions an accompanying goitre. Duchosal and Junet (1926) studied a single neotenic specimen of *Triturus* ("Triton") *alpestris* and reported that the thyroid gland did not differ in histological appearance from that of a normal specimen. Kuhn (1925) studied the thyroid glands of a single neotenic specimen of *Triturus cristatus*: he found the glands to be normal in size, though consisting of more numerous, smaller follicles than is usually the case in normal specimens. Hartwig and Rotmann (1940), in an extensive study of a partially neotenic population of 'Triton taeniatus' (*Triturus vulgaris*), state that the neotenic specimens which they examined had thyroid glands of normal size but showing some histological signs of low activity. They also point out, however, that the histological

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appearance of normal newt thyroids varies considerably through the year and that there is no sharp distinction between neotenic and normal specimens as regards the histological appearance of these glands. Hartwig and Rotmann furthermore give a comprehensive review of the findings of previous workers on urodele neoteny: the same subject has also been reviewed by Wolterstorff and Freytag (1951) and by Lynn and Wachowski (1951): nowhere has goitrous enlargement of the gland been encountered.

Not only have goitres never been reported from urodeles in nature: experimental work testing various goitrogens has shown that both urodeles and anurans are highly resistant to the goitrogenic activity of these substances. Joel, D'Angelo, and Charipper (1949) have pointed out that the changes in amphibian thyroids induced by various goitrogens are both qualitatively and quantitatively less marked than those which occur in birds and mammals as a result of similar treatment. Adams (1946) kept adult specimens of *Triturus viridescens* in strong solutions of thiourea for 86 days and found only slight hyperplasia. In view of these findings the occurrence in nature of neotenic newts with accompanying goitre is of particular interest.

MATERIAL

In a routine collection of newts from a pond near Crail, Fife, on 19 May 1951, two neotenic specimens of *T. helveticus* Razoumowsky were noticed. One was a female, length 7.1 cm., the other a male, length 6.5 cm. Both specimens were in full breeding dress, yet with persistent larval gills and larval head shape. Both specimens died within 3 days of capture, probably owing to their having been kept in damp moss instead of water. Neither of these newts showed evident external signs of goitre and histological examination was not attempted.

In a further collection of newts from the same pond on 14 May 1952, two more neotenic specimens of *T. helveticus* were obtained. One animal, length 6.7 cm., with well-developed male secondary sexual characters, showed a pronounced bilobed pink swelling in the throat region lying below the operculum and extending beyond it posteriorly. A photograph of this animal (specimen A) is shown in fig. 1, A. The swelling was diagnosed as a goitre and subsequent histological examination confirmed this diagnosis. The other neotenic specimen from this collection, a male, length 5.2 cm. (specimen B), had no goitre.

Subsequently three further neotenic goitrous specimens (C, D, and E) of *T. helveticus* were obtained from the same pond on 11 July 1952. All three animals were females, their respective overall lengths being 7.0, 7.5, and 7.0 cm. Photographs of specimens D and E are shown in fig. 1, B-D.

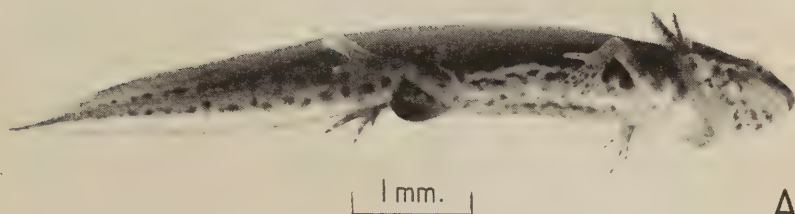
During 1953 the pond was visited on numerous occasions and several hun-

FIG. 1 (plate). A, specimen A, showing goitre, persistent gills, and male secondary sexual characters.

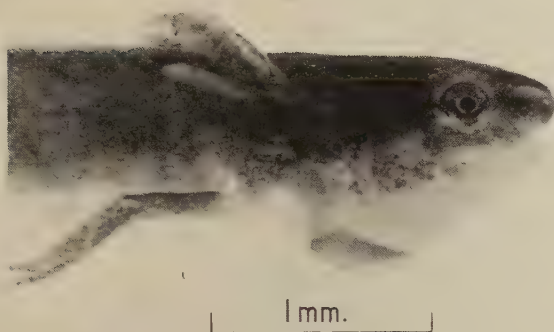
B, specimen D, profile view.

C, specimen D, ventral view showing asymmetrical goitre.

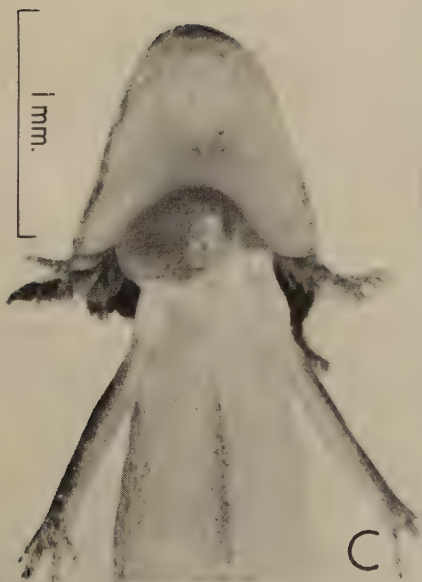
D, specimen E, ventral view showing smaller bilateral goitre.



A



B



C



D

FIG. 1
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dred newts were examined. Throughout the year 1953 and up to the present time in 1954, neither neotenic nor goitrous newts have been found.

All the labelled neotenic specimens were fixed in Bouin's fluid and the entire lower jaw of each was serially sectioned to the level of the posterior margin of the thyroid glands. The histology of the glands is described in the next section. Owing to an unfortunate accident the pituitary glands of these animals were not subjected to histological examination.

The pond from which these newts were obtained lies in a deep pear-shaped pit in heavy soil. It is approximately 100 feet long by 50 feet wide at the widest point. The pit was originally excavated as a stone quarry but the sides, steeply sloping for the most part, are now covered with soil and heavily overgrown with vegetation. No figures are available as to depth, though this is evidently considerable and may well reach 15 or more feet. The water surface, which shows marked fluctuation in level from one season to another, lies some 25 feet below the level of the surrounding agricultural land.

A thriving rabbit colony inhabits the sides of the pit and feeds in the surrounding fields. These fields are all under cultivation, turnips and kale being grown as part of the normal rotation. It is suggested later that this may have some bearing on the occurrence of the goitre.

MORPHOLOGICAL AND HISTOLOGICAL DESCRIPTION OF THE THYROID GLANDS

Normal and neotenic non-goitrous specimens

The thyroid glands of normal adult newts are paired structures lying immediately anterior to the arterial arches and lateral to the genio-hyoideus muscles. They are spheroidal or ellipsoidal bodies, the longest dimension varying between 300 and 700 μ . They consist of a few large follicles and have no distinct capsule. The follicular epithelium varies through the year from squamous to cubical, and the degree of vacuolation of the colloid also varies.

Specimen B is neotenic but non-goitrous. Its thyroid glands (fig. 2, A) lie within the size range of those of normal newts of similar size (600 by 560 μ), but they show signs of low activity. The central section of a complete series shows seven large follicles full of eosinophil colloid, which is poorly vacuolated. The follicular epithelium is low and the cell boundaries are difficult to make out.

Neotenic goitrous newts

The goitres described here have many features in common though they vary considerably in size, the largest extending from mid-eye region to the level of the heart and causing great distension of the entire throat. All are markedly hyperaemic, the hyperaemia being readily visible through the operculum, whose epidermis, though semi-transparent, is flecked with yellow pigment. The goitres result in major displacement of structures in the throat region, though there is no invasion of other tissues such as has been found in fish thyroid gland tumours. The goitres are not encapsulated, but their

boundaries are well marked and distinct. The follicular epithelium is not folded: mitotic figures are not common. The colloid is variable in staining reaction in different parts of the gland: vacuoles are small and few in number.

Specimen A (figs. 1, A and 2, B) showed the largest goitre in the series. The goitre is symmetrically bilobed, each lobe measuring approximately 4 by 3.5 mm. With the exception of the genio-hyoideus muscles and the skeletal structures it occupies the entire throat region. The follicles are more or less uniform in size and are smaller than those found in a normal newt thyroid. Cytological details cannot be described since the specimen was fixed after death.

Specimen C also showed a bilobed goitre, the dimensions of each lobe being approximately 2 by 2.3 mm. The follicular epithelium varies from squamous to high columnar: there are few large follicles with low epithelium and abundant poorly vacuolated colloid, and many small follicles with high columnar epithelium and little or no colloid. The colloid varies in staining reaction. In the large follicles it takes eosin and Heidenhain's haematoxylin, whereas in all the small follicles it takes Heidenhain's haematoxylin exclusively (fig. 2, D). A few cellular inclusions are present in the colloid. The large follicles are irregular in shape and elongated in diverse planes: such follicles are reminiscent of the normal thyroid gland, having low epithelium, indistinct cell boundaries, and colloid with few vacuoles.

Specimen D (fig. 1, B, C; fig. 2, C, E, F). The thyroid gland on the right-hand side of this specimen measures 4 by 4.5 mm. and is much larger than that of the left-hand side (1.4 by 1.6 mm.), though both are goitrous. The larger gland contains many small follicles, each with high columnar epithelium and very small lumen: some regions are non-follicular. There are no cell inclusions in the colloid. The follicles of the smaller gland are larger, the follicular epithelium varying between cubical and squamous. A large number of small peripheral vacuoles are present in the colloid.

Specimen E (fig. 1, D; fig. 2, G). The goitre in this specimen is smaller than

FIG. 2 (plate). A, specimen B, T.S. lower jaw showing thyroid glands of normal size. Bouin fixation; iron haematoxylin and orange G / erythrosin.

B, specimen A, T.S. lower jaw showing massive goitre. The area at top centre consists of the genio-hyoideus muscles. Bouin fixation after death; iron haematoxylin and eosin. The operculum of this specimen was dissected away before fixation.

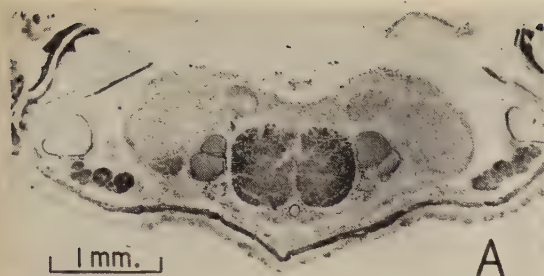
C, specimen D, T.S. lower jaw showing asymmetrical goitre. Bouin fixation; iron haematoxylin and orange G / erythrosin.

D, specimen C, T.S. of part of lower jaw showing goitre. Bouin fixation; iron haematoxylin and eosin. Two regions can be differentiated, one in which the follicular epithelia are low and the colloid eosinophil, the other with higher epithelia and the colloid stained with haematoxylin.

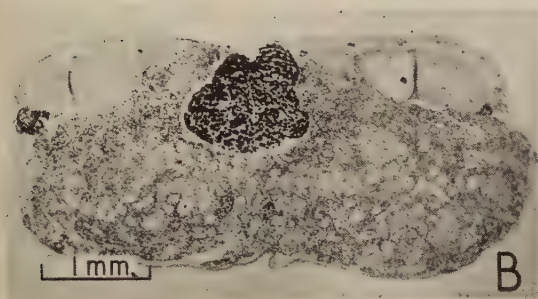
E, specimen D, T.S. of non-follicular and microfollicular regions of goitre. Bouin fixation; iron haematoxylin and orange G / erythrosin.

F, specimen D, T.S. of single follicle. Bouin fixation; iron haematoxylin and orange G / erythrosin.

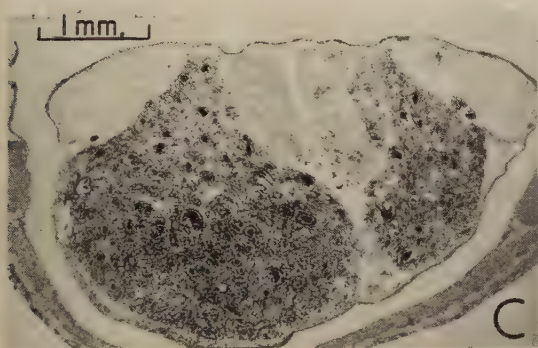
G, specimen E, T.S. of single follicle showing low epithelium and cellular inclusions in the colloid. Some of these included cells appear to be in the process of division. Bouin fixation; Mallory's triple stain.



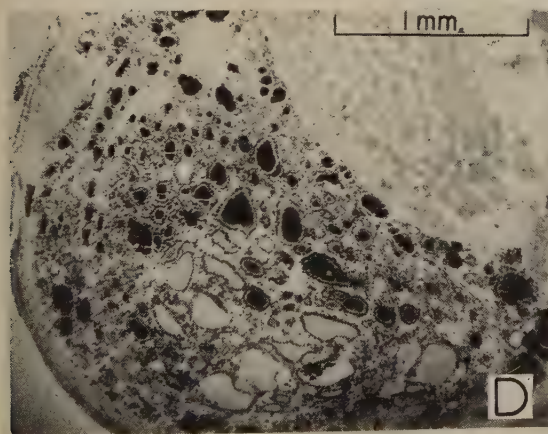
A



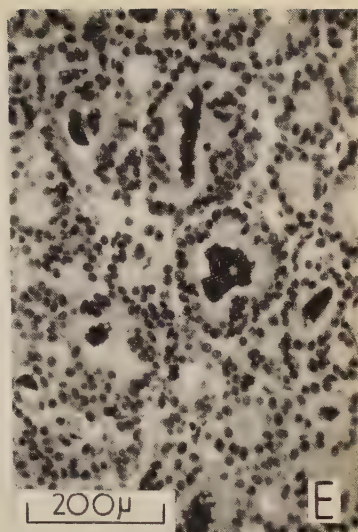
B



C



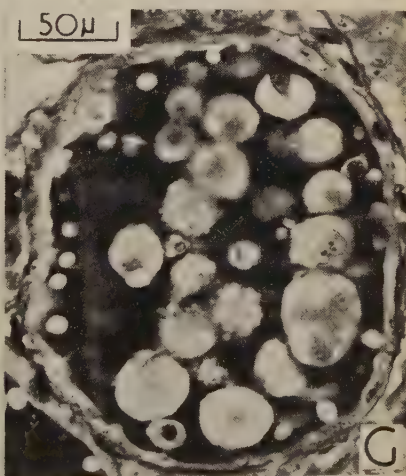
D



E



F



G

FIG. 2

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those of the preceding specimens (approximate dimensions of each lobe, 1.7 by 1.8 mm.): it is symmetrically bilobed. Each gland has a relatively normal looking medio-ventral region with large follicles having eosinophil colloid and cubical to squamous epithelium. There are few non-follicular areas. Many of the follicles have colloid in which there are large spheroidal vacuolated cells with nuclei similar to those of the cells of the follicular epithelium itself.

STATUS OF THE GOITRES

As already mentioned in the introduction, previous authors have described few or no histological signs of abnormality in the thyroid glands of neotenic newts. The thyroid glands described in the present paper, on the other hand, are evidently extremely hyperplastic and they appear to have been under the influence of an abnormally high level of circulating thyroid-stimulating hormone. Though there is no invasion of foreign tissue, the glands are so massive as to have caused gross displacement of neighbouring structures in the lower jaw. Marine and Lenhart (1910) have argued at length as to the status of so-called thyroidal carcinomata encountered in trout: in spite of the fact that the trout tumours invade bone, muscle, and other tissues, these authors have concluded that the tumours merely constitute an extreme example of endemic goitre and that they are not truly cancerous. The newt goitres resemble closely in histological appearance the spontaneous thyroidal growths described by Gorbman and Gordon (1951) in *Xiphophorus montezumae*, and we consider that they should in all probability be similarly classed as thyroidal tumours of a benign nature.

THE NEWT POPULATION

Although we can give no accurate idea of the size of the newt population in the pond at Crail, this certainly numbers several hundred adults and may well run into thousands. The population is mixed and includes both *T. helveticus* and *T. vulgaris*. Collections made in March or April include only 10 per cent. or less of *vulgaris*, but the *vulgaris* component of the population increases during the spring and by June the two species appear to be almost equally common. Towards the end of the breeding season the relative abundance of *vulgaris* declines. *T. vulgaris* in most localities in Britain leaves the water at the end of the breeding season and hibernates terrestrially: clearly the habits of *vulgaris* in this pond are those normal for the species.

T. helveticus is considered to be more aquatic in its habits than *T. vulgaris* (compare Smith, 1951, p. 65). Many, though not all, of the *helveticus* inhabiting the Crail pond overwinter in the water and we have collected them as late as November in large numbers and in full breeding dress. Collections in March, however, although consisting for the most part of fully aquatic specimens in good condition, include also a small proportion of specimens which have lately returned to the water, these being recognizable by the rough texture of their skins. Overwintering larvae are common and in spring collec-

tions these show marked variation in size, some being presumably in their first and some in their second year of life.

The neotenic and goitrous newts which we have collected all belong to the species *T. helveticus*. Such specimens form not more than 1 or 2 per cent. of the total number of *helveticus* which we have examined. Since most of our large-scale collections have been made in April or May we have handled far fewer *vulgaris* from this pond. This may account for our failure to find neotenic and goitrous specimens of *vulgaris*: alternatively such animals may not be present in this population.

DISCUSSION

Many theories have been put forward in attempts to account for the occurrence of neoteny in urodele Amphibia. We do not propose to review the subject exhaustively in the present paper since extensive surveys have already been made by Hartwig and Rotmann (1940) and by Lynn and Wachowski (1951). Most theories postulate dysfunction of the thyroid and/or pituitary glands, due to intrinsic or extrinsic agents.

No student of urodele neoteny has succeeded in providing histological evidence of pituitary dysfunction, but several other lines of evidence converge to implicate the pituitary in this phenomenon. In Anura the important role played by the pituitary in metamorphosis has been known for many years (Allen, 1916). The experiments of Reineke and Chadwick (1939) on *T. viridescens* have demonstrated that the urge to enter the water habitat is dependent on a hormone produced by the anterior lobe of the pituitary. Blount (1939) caused the metamorphosis of the normally neotenic *Amblystoma mexicanum* by implantation of pituitary rudiments from the normally metamorphosing *A. tigrinum*. As a consequence of this and later work (Blount and Blount, 1947), it has been claimed that two types of thyroid-stimulating hormone are produced by the pituitary, one being responsible for the production and storage of thyroid secretion, the other concerned with its release. Blount and Blount suggest that in neotenic forms the releasing hormone is absent, but the evidence is as yet by no means conclusive. The literature on neotenic newts contains frequent references to specimens which are partial albinos. Partially albino newts are rare: so are neotenic newts. Clearly there is a correlation between these two conditions (Smith, 1951) and the pituitary gland may conceivably be the common factor.

Above all, the pituitary gland's connexion with neoteny has been frequently postulated on account of the controlling influence which it is known to exert over the thyroid; and knowledge of the implication of the thyroid itself in amphibian metamorphosis dates from the classical experiments of Guderhatsch (1913) and Allen (1916) on *Rana spp.* and of Jensen (1916) and Huxley and Hogben (1922) on *Amblystoma*. The present observations substantiate this connexion: neotenic newts are rare; goitres have only been observed in neotenic specimens. These two phenomena are clearly correlated with one another.

Any attempt to account for neoteny in newts is faced with the problem of the coexistence in the same pond of neotenic and normal specimens. All recorded cases are similar in this respect. Several authors have consequently been led to postulate a genetic origin of neoteny. In all experiments where neotenic newts have given rise to offspring, including a successful mating of a virgin neotenic female with a neotenic male *T. vulgaris*, the offspring have metamorphosed as rapidly as have normal control larvae. Although there may be a genetic component determining a tendency to neoteny, this will be most difficult to demonstrate owing to the well-known acceleration of metamorphosis which regularly attends the raising of newt larvae in captivity.

Several authors have attempted to correlate the occurrence of neoteny with peculiarities of habitat, but Hartwig and Rotmann, in reviewing the literature on this subject, were unable to find any significant factors common to ponds with neotenic newt populations. Nevertheless there are two distinct indications that habitat peculiarities may act as causative factors. Zeller (1899) found neotenic specimens of three different species, *T. vulgaris*, *T. alpestris*, and *T. cristatus* in one and the same quarry pond, while Smith (1950) has described two ponds containing neotenic newts together with giant anuran tadpoles.

Low iodine concentration in the environment is one of the best authenticated causes of goitre. In the present instance, however, although no iodine determinations have been made for the water of the pond in question, it seems unlikely that lack of iodine could be the cause since the pond lies within two miles of the sea and in a district where extensive use is made of sea-weeds as fertilizers. Furthermore the iodine content of Crail drinking water, which is taken from a nearby reservoir, is given as 5 mcg. per litre by Murray and others (1948). This is the second highest iodine concentration recorded in determinations for 64 Scottish localities.

We are led to postulate that the thyroid goitres here described were produced by the action of a naturally occurring goitrogen, the so-called 'brassica factor' (see review by Lever, 1951). The goitrogenic action of a diet of cabbage on rabbits was first demonstrated by Chesney and others (1928) and the active substance isolated and synthesized by Astwood and others (1949). The latter authors identified the 'brassica factor' as L-5-vinyl-2-thiooxazolidone.

Our evidence in favour of this hypothesis is based on four considerations. First, the land surrounding the Crail pond is extensively cultivated: the crop rotation practised includes yellow turnip and kale, and these plants were grown in fields bordering the pond in 1951 and 1952 but not in 1949, 1950, or 1953. Neotenic newts were collected in 1951, neotenic and goitrous newts in 1952, but neither in 1953 or 1954. Secondly, rabbits which have their burrows in the slopes leading down to the pond feed in the surrounding fields. Rabbit faeces accumulate in large quantities on the slopes and rainwater carries faeces and extract of faeces into the pond by drainage. It might reasonably be expected that this drainage water should contain the 'brassica factor' at times when the rabbits are feeding on turnips and kale. Thirdly, the majority of the larvae and adults of *T. helveticus* overwinter in the water of the

Crail pond and hence are more exposed to any goitrogens which may be present than are newts which habitually overwinter on land. Fourthly, although adult amphibians are known to be highly resistant to goitrogens, we do not know how larval and metamorphosing animals are likely to react. From unpublished work on metamorphosing *Xenopus* larvae in which extensive goitres have resulted from thyroidectomy (possibly due to the hyperplasia of supernumerary follicles) it would appear that the thyroid is in a particularly sensitive state at the time of metamorphosis. Should this be true also of newts, the overwintering *helveticus* larvae might well be exposed to the postulated goitrogen at this critical time.

We have no experimental evidence in support of the above hypothesis. Crude extracts of seeds of *Brassica spp.* have unfortunately proved highly toxic to both larval and adult newts and in further work we must test the activity of the purified 'brassica factor'. In our hands 0.05 per cent. thiourea and 0.01 per cent. 2-thiouracil have failed to prevent metamorphosis and have also failed to produce goitres in newt larvae taken from the Crail pond.

This study forms part of a research programme in comparative endocrinology which is supported by a grant from the Nuffield Foundation. Our thanks are also due to Mr. D. R. R. Burt and Mr. M. D. B. Burt who introduced us to the pond at Crail and who captured the first neotenic specimens.

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Crystalline Properties of Spicules of *Leucosolenia complicata*

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With 3 plates (figs. 1, 2, and 4)

SUMMARY

Spicules of *Leucosolenia complicata* acquire crops of crystals when left in calcium bicarbonate solution. The sites occupied by the crystals depend on the type of prior corrosion which the spicules have undergone. Thus spicules isolated by 5 per cent. potash solution develop crystals on the surfaces transverse to the optic axis (basal rays and slender monaxons excepted), those isolated by potassium nitrate solution on the surfaces parallel to the optic axis. It appears that on these surfaces respectively rhombohedral and prismatic faces are corroded and enlarged, thereby facilitating the subsequent crystallization upon them. Continued prior corrosion leads to the spread of the crystal sites around the rays.

The crystals are calcite rhombohedra and all bear the same crystallographic orientation as the calcite of the spicule on which they have crystallized. The relationship between the crystal faces and the spicule rays is a guide to the orientation of the spicule with reference to the crystallographic axes of calcite, and provided it be assumed that the crystals are positive rhombohedra, agreement has been obtained with von Ebner's deductions, based on corrosion phenomena.

A discussion is given on the factors controlling spicule form, particularly with respect to the uniplanar growth of the rays and the planar angle. The facts suggest that the crystallizing solution favours crystallization mainly on the pair of surfaces transverse to the optic axis, and the curvature in the plane containing the optic axis is caused by the choanoderm deflecting the calcoblast on the ray tip and thereby hindering the crystallization on one or other surface. The elliptical cross-section of the rays (basal rays and slender monaxons excepted) indicates the same preferential crystallization. To explain the planar angle it seems necessary to postulate the existence of a spiralling submicroscopic fibrillar system in the oscular tube, the fibrils being parallel to the spirals on which the spicules and pores tend to lie. A preliminary account of the spiral organization of the tube is given.

It is concluded that the spicule form can be explained by a combination of extrinsic and intrinsic factors, the latter depending on the properties of the crystallizing material, the former on the gene complex.

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INTRODUCTION

CALCAREOUS sponge spicules are composed largely of calcite (Sollas, 1885; von Ebner, 1887) and behave optically as single birefringent crystals with a crystallographic structure oriented in a definite way with respect to the form of the spicule. Von Ebner deduced this orientation from a study of the corrosion patterns produced by formic acid on the surfaces of giant spicules of species of *Leucandra* and *Leucaltis*, and also investigated the orientation of the optic axis in other species. He found that the spicule rays bear a constant relationship to the horizontal crystallographic axes of a calcite crystal and was able to classify the spicules into types according to the relationship. It had also been shown previously by Sollas (1885) that the optic axis had a definite orientation in spicules of *Grantia compressa*, and the author has investigated the change in orientation with position in the oscular tubes of *Leucosolenia complicata* (Jones, 1954a).

For a calcite crystal the three horizontal axes are inclined at 120° to each other in the plane perpendicular to the vertical (optic) axis and Bidder (1898) attempted to explain the alate form of the triradiates of *L. lieberkühnii* by assuming that the calcite tended to crystallize in the three planes containing the optic axis and respectively these three horizontal axes, the actual direction of growth in each plane being determined by the limitations imposed by the mesogloecal boundaries of the oscular tube. However, the author (Jones, 1954b) has shown that while there does seem to be a tendency for triplanar growth in triradiates, the three planes used by the rays of *L. complicata* spicules are not inclined at 120° to each other, and that the angle between the oscular ray planes (the planar angle) is unlikely to be controlled by the crystallization process solely. On the other hand, the shapes of aberrant spicules (fig. 1, A-D) often emphasize the existence of a 'crystallomorphic' factor influencing spicule form, as Bidder suggested.

A note by Sollas (1886) describes the formation of tiny crystals on calcareous sponge spicules that had been left in a solution containing 'excess calcium carbonate'. These crystals were confined to the extremity of the basal ray and to the two opposite edges, 'front' and 'back', of the paired rays, while furthermore they had the same crystalline orientation as had the spicule on which they had crystallized, the whole extinguishing simultaneously when rotated between crossed nicols. The form of such crystals would obviously be a good guide to the crystallographic structure of the spicules, while their arrangement about the spicule surface might throw light on the properties of crystallizing calcite and hence on the crystallomorphic factor. For these reasons the crystallization experiments described below were attempted.

It should be borne in mind that the spicules do not consist solely of calcite, but possess an axial filament along the centre of each ray, and a sheath, both probably of organic matter (Minchin and Reid, 1908). After isolation by strong potash solution, however, the sheath contains inorganic matter, probably calcareous (Bütschli, 1901, 1907). The calcite itself is impure, small quantities of sulphate and magnesium being present.

METHODS

Spicules of *L. complicata* (Minchin, 1904) were isolated from clean oscular tubes fixed in 90 per cent. alcohol by two methods. In the first the material was placed in a boiling tube containing 5 per cent. potassium hydroxide solution and this was heated for about 5 minutes in a bath of boiling water (excessive contact with the potash should be avoided as it corrodes the spicules). The residue was vigorously washed twice with water with the aid of a centrifuge and the liberated spicules were suspended in absolute alcohol. With the second method freshly collected living oscular tubes were brushed and then placed in a solution containing 5 per cent. potassium nitrate, which disperses the intercellular 'cement' and mesogloea after about $1\frac{1}{4}$ hours (Jones, 1952). The disintegrated mass was then shaken up in the solution and the freed spicules washed rapidly in 70 per cent. alcohol before suspending in absolute. A few drops of either suspension were then spread over a slide and allowed to evaporate, a fine brush being used before the slide was dry to separate any spicules that had clumped together. The spicules thereby become attached to the slide and are not dislodged if it be subsequently dipped into solutions in staining jars.

Since calcium carbonate is so sparingly soluble in water it is impracticable to use it directly for the crystallization experiments. A solution of calcium bicarbonate, however, continuously generates the ions of calcium carbonate by the release of carbon dioxide to the air, and in consequence makes an excellent substitute. Such solutions were made up by warming a suspension of 5 gm. pure calcium carbonate in 300 ml. distilled water to about $70^{\circ}\text{C}.$, and then bubbling carbon dioxide through from a cylinder for 3 minutes. The suspension was allowed to settle and was then filtered to remove calcite crystals which would compete with the spicules for the ions.

If 2 ml. of such a solution are spread completely over a slide of spicules, placed on a hot plate ($25\text{--}30^{\circ}\text{C}.$), crystal crops will be obtained on the spicule rays and will be well developed after about 1 hour. The excess solution may then be drained away and the slide taken up through distilled water and 90 per cent. alcohol to absolute (twice) and finally allowed to dry. A permanent preparation may be made with synthetic neutral mountant or DPX, in which media the examination of the spicules is facilitated if a piece of polaroid is inserted beneath the microscope condenser and rotated until the spicule is most distinct. For the orientation of the crystal faces, however, it is better to examine the spicules in air.

The pH of the filtered solution is usually about 7.0 (measured by using indicators), but observations on the formation of crystals on spicules lying in a Petri dish of 3-inch diameter containing 20 ml. crystallizing solution have shown that crystals do not appear until the pH is about 8.0. This pH is of course fairly rapidly reached when the solution is spread over the slide. However, a solution that has been allowed to stand until after it has this pH and has been re-filtered is sometimes unable to produce good crystal crops, but does so if the slide is first dipped into a weak carbonic acid solution

(pH 6.0–6.5) at room temperature for a minute or so, and then rinsed and dried. This slight prior corrosion facilitates the initiation of crystal development by the weakened solution.

For some experiments solutions of calcium bicarbonate with a starting pH of less than 7.0 were used. Such solutions were prepared as above and filtered, but divided into two, one half being subjected to further bubbling with carbon dioxide until the pH was about 5.5 and the two halves then being recombined in suitable proportions to give the desired starting pH.

To investigate the effect of prior corrosion on the spicules, a uniform series of slides was prepared and numbered. The second, third, &c., slides were then immersed in carbonic acid solution for intervals progressively increasing with the series, and then rinsed, dehydrated, and dried. The first slide was untreated and used as a control. Each slide was next given 2 ml. of calcium bicarbonate solution that had been allowed to stand in an open dish until the pH after filtering was at least 8.0. After $\frac{3}{4}$ –1 hour the slides were all rinsed once more, dehydrated, dried, and mounted in the usual way. The results depend on the intervals selected and on the pH of the carbonic acid solution used.

In order to determine the siting of the crystals accurately, spicules with crystals were mounted on the tips of finely drawn out glass needles as described by the author (Jones, 1954a) and rotated into the appropriate settings for observation.

RESULTS

Sites occupied by the crystals on spicules isolated by KOH

Sollas (1886) noted that crystals he had accidentally obtained were restricted to the opposite sides of the paired rays and the extremity of the basal ray, and such has largely been found with the experiments concerned here. However, the number of crystals is dependent to a great extent on the amount of prior corrosion suffered by the spicules (see below), and it is more usual using the method outlined above for the basal ray to acquire its crystals in four rows all along its length, each row being roughly half-way between the central axes of the lateral and the gastral or dermal surfaces respectively (figs. 1, E, F, and G; 2, B and F). Sometimes extra crystals are distributed more at random

Fig. 1 (plate). A and B, aberrant triradiates of *Clathrina* (= *Leucosolenia*) *coriacea*. Note the angles of 60° or 120°, which suggest control by a crystallomorphic factor.

C, aberrant triradiate of *L. variabilis*. Note the apparent development of faces at the edges and the parallel course of the oscular rays. The direction of growth of the oscular rays in general cannot depend on the initial arrangement of the cells in the formative sextet, since here the cells must have been widely separated when they set out on their parallel courses.

D, aberrant spicule of *L. variabilis*, exhibiting crystal-like faces along the edges.

E, mounted quadriradiate of *L. complicata*, set for side-view observation. The lateral surfaces of the basal and gastral rays are free from crystals.

F and G, tri- and quadriradiate of *L. complicata* respectively, showing the centro-lateral rows of crystals on the gastral surface of their basal rays, and their continuation with the 'back' crystal rows on the oscular rays.

In each photomicrograph the line represents 50 μ .

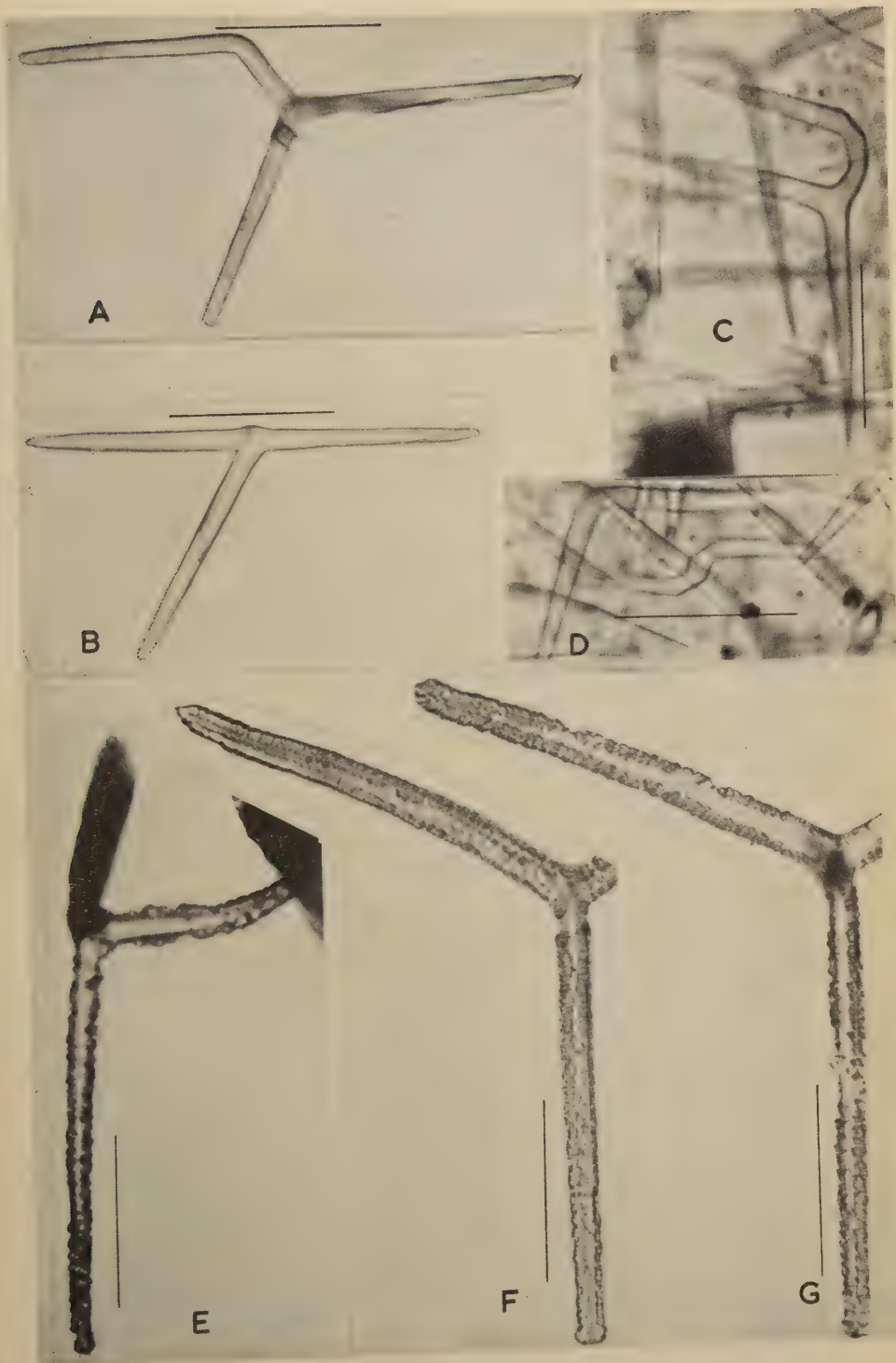


FIG. 1
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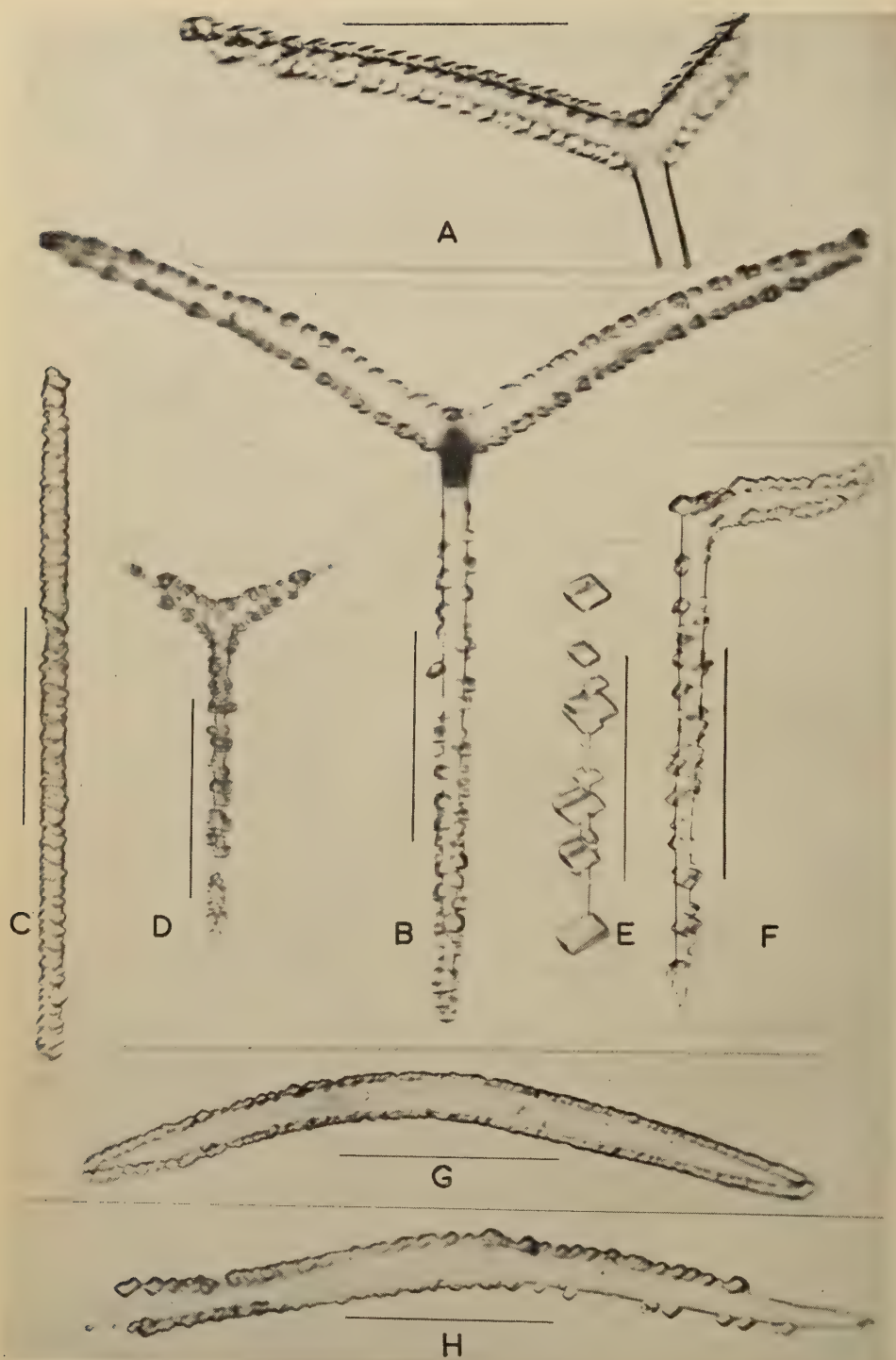


FIG. 2
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on the gastral or dermal surfaces, and occasionally they also occur on the lateral surfaces of the basal ray.

The crystal rows on the oscular rays appear at first sight to lie along the front and back edges (the back edge facing in the direction of the basal ray tip), but closer inspection reveals that the front row lies in a higher focal plane than the back when the spicule is resting on the tips of its rays, and that the plane containing the two rows is inclined slightly to the basal ray. By setting mounted quadriradiates with crystals so that their optic axis coincides with the line of sight, the crystal rows are seen to lie centrally along the top and bottom surfaces of the oscular rays (fig. 3, C), hereafter termed the 'o.a.-transverse' surfaces. The same is true for the gastral rays; the crystals lie on the top and bottom surfaces when the optic axis is vertical.

In well developed examples the basal ray rows of crystals join up with those of the oscular rays, the rows on the back edges of the latter continuing on each side into a gastral centro-lateral row of the former (fig. 1, F and G), and those of the front edges into the dermal centro-lateral rows; the latter diverge gradually as they cross the dermal surface of the oscular rays. The gastral ray crystals likewise merge with the gastral rows on the basal ray behind and sometimes broaden out from the front row to meet the front oscular ray rows.

The lance-headed monaxons, as would perhaps be expected from their similarity to the oscular rays of tri- and quadriradiates (Sollas, 1885), also acquire their crystal crops in rows along the top and bottom edges when the optic axis is vertical. Since these spicules are curved in the plane containing the optic axis, they usually lie on their o.a.-parallel sides on the slide and the crystals are then seen along the edges (fig. 2, G and H). With slender monaxons the crystals develop in typically one row near the centre of the surface not resting on the slide, though sometimes two or more rows can be discerned (fig. 2, C and E).

Only 10 seconds' immersion in carbonic acid (pH 5.0) may be sufficient to alter the crystal picture, and with increasing periods of such prior corrosion the spicules develop progressively more, and hence smaller, crystals in the same time. The crystals become more numerous in the zones described above, forming a band instead of a row along the o.a.-transverse surfaces of the oscular rays, but with additional prior corrosion crystals are developed farther and farther round the rays until they litter the o.a.-parallel surfaces. This

Fig. 2 (plate). *L. complicata* spicules with crystals.

- A, triradiate with well developed crystal rhombohedra along the 'front' and 'back' edges.
 - B, quadriradiate with similarly arranged crystals and also centrolateral rows on the basal ray.
 - C, slender monaxon with closely crowded crystals.
 - D, triradiate showing the same pattern of crystals as the normal spicules even after considerable prior reduction in size by corrosion with acid alcohol.
 - E, slender monaxon with a few well-developed crystal rhombohedra.
 - F, quadriradiate in side view. Note the centro-lateral rows on the basal ray and the absence of crystals on the lateral surface of the gastral ray.
 - G and H, lance-headed monaxons with crystals on the surfaces transverse to the optic axis.
- In each photomicrograph the line represents 50 μ .

result is most easily seen when the crystallization has proceeded for about $\frac{1}{2}$ – $\frac{3}{4}$ hour; with longer periods (1 hour) the numerous tiny crystals fuse up to form a 'skin' over the ray surface. On the basal ray the crystal sites are likewise opened up all over the surface by the prior corrosion and continued crystallization results in the formation of broad crystals spreading across the top and bottom surfaces, the chevron-like appearance being similar to that often seen with the slender monaxons (fig. 2, c). The crystals also develop on the lateral surfaces of the gastral rays and of curved monaxons after prior

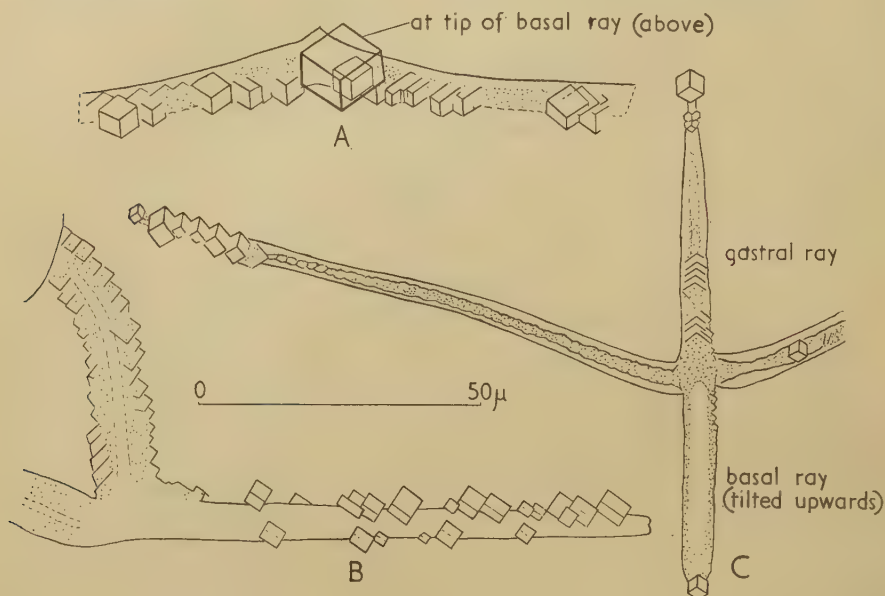


FIG. 3. Camera lucida drawings of mounted spicules of *L. complicata* showing the arrangement and form of the crystals. Where the crystal form is poorly distinguishable the siting has been indicated by the dotted area. A, triradiate in transverse view. B, quadriradiate in side view. C, quadriradiate in planar view (optic axis coincident with line of sight), the gastral surface of the basal ray being uppermost. The crystals lie on the top surface of the oscular and gastral rays.

corrosion. However, even when the spicules are very heavily corroded (by acid alcohol or carbonic acid) and considerably reduced in size, it is possible to obtain a neat crystal pattern with the crystals mostly restricted to a single row on the o.a.-transverse surface of the oscular rays (fig. 2, d).

Sollas (1885) had already observed that corrosion was most marked on the 'front' and 'back' (really the o.a.-transverse) edges of the oscular rays when *Grantia compressa* spicules were left in balsam or dilute acetic acid, and he concluded (1886) that the crystals were deposited in regions most liable to solution. The spread of the crystal sites with increasing corrosion is thus presumably due to the etching out and enlargement of suitable faces on the surface of the spicule calcite, and since the corrosion area will tend in time to spread from the most easily corroded zone, crystal sites will be exposed

farther and farther round the rays. The limits of the corrosion area will, however, offer the best sites for crystal growth if the supply of ions (i.e. the degree of supersaturation) is limited, for at the edges the ions can approach from a larger volume of solution, whereas more centrally the competition for ions will be greater. Thus larger crystals should develop at the edges and as they grow they should trap more of the ions appearing in the intermediate zone, since these ions will tend to diffuse down the concentration gradient towards the region of crystallization and thus bring about a reduction in the degree of supersaturation in that zone. This explains why a double row of large crystals is quite often obtained on the o.a.-transverse surfaces of curved monaxons and oscular rays and may help to explain the development of two centro-lateral rows on the top and bottom surfaces of the basal ray.

The need for prior corrosion and the fact that when few crystals are developed they are relatively large suggest that crystal initiation is not as easy a process as crystal growth. This was confirmed by placing a slide of spicules in a Petri dish of $3\frac{1}{2}$ -inch diameter containing 70 ml. of filtered crystallizing solution (pH 7.0) and leaving it undisturbed for 40 hours at 13.5° C. After this time few of the spicules had crystal crops and their crystals were large, of rather irregular shape and siting, and few in number, whereas the slide was littered with free crystals that had clearly not settled down from the surface of the solution since at one place they had crystallized along the edges of a scratch on the glass surface. Thus at the greater depth below the surface of the solution, crystal formation and growth are not prevented, given time, but the lower degree of supersaturation of the calcium and carbonate ions caused by the slow diffusion of carbon dioxide through the solution (a similar solution tinted with BDH universal indicator in a test-tube rapidly shows a stratification of colours) has hindered the initiation of crystal development on the spicule surface. Thus the degree of supersaturation is an important factor controlling crystal initiation and for good crystal crops it is necessary to have the spicules near the surface (as when 3 ml. of the solution are spread over the slide). Actually it has been found that the solution usually employed can be diluted to half-strength and still yield crystal crops on the spicules.

So far the possible influence of the sheath has been neglected and it might be supposed that the hindrance to crystal initiation is due to the presence of the acid-soluble inorganic component which Bütschli (1901) isolated as an artifact from the monaxons of *Leucandra aspera* with 35 per cent. potash solution. The prior corrosion described above might conceivably dissolve this sheath progressively from the o.a.-transverse zones, thereby opening up the crystal sites. However, the immersion of slides of spicules in 10 per cent. potash solution for 15 minutes, followed by rinsing and drying, resulted in an increase in the number of subsequently utilized crystal sites when the results were compared with the control slides, whereas if the sheath produced by the action of the potash were hindering crystal initiation, a decrease would have been expected. It thus seems unlikely that the few minutes' boiling with

5 per cent. potash solution when isolating the spicules will have produced a sheath capable of preventing crystal formation. Furthermore, the preferential crystallization on the o.a.-transverse surfaces of the oscular rays of heavily corroded spicules (fig. 2, D) likewise shows that a selective corrosion of an inorganic sheath is not determining the position of the crystals, since the inorganic sheath must be absent if it is invisible in spicules considerably reduced in size, since it is non-contractile. It is also unlikely that the crystals are developing on the organic component of the sheath (Minchin and Reid, 1908) as this will be distorted in such corroded spicules. The crystals are firmly attached to the rays and cannot be removed by teasing the spicules with needles, another indication perhaps of the direct connexion between the calcite of the rays and the crystals. It thus appears that the organic sheath is perforated and probably affords the main hindrance to the exploitation of crystal sites on the ray calcite; further evidence on this point will be given in a later paper.

Crystal pattern with spicules isolated by KNO_3

Spicules isolated by means of 5 per cent. potassium nitrate solution (pH 7.0) develop crystals in a pattern complementary to that formed by the same crystallizing solution with KOH-isolated spicules, for the crystals arise on the o.a.-parallel surfaces of the rays. If the spicule has not been in contact with the nitrate solution for long ($1\frac{1}{4}$ hours), few crystals develop and these sometimes show a symmetrical arrangement, a single crystal forming at each oscular ray tip and a single one about half-way along each oscular ray, with a few crystals at the basal ray tip. After longer immersion (2 hours) the spicules acquire good crops with the crystals forming a row or band along the o.a.-parallel surfaces of the oscular, gastral, and basal rays, and of the curved monaxons (fig. 4, A, B, C, D, and E). The slender monaxons, however, give the same picture as described above (fig. 4, F and G). Crystals also appear

Fig. 4 (plate). *L. complicata* spicules

A and B, crystals on the o.a.-parallel surfaces of the oscular and basal rays of respectively a quadri- and a triradiate. Compare with fig. 2, B.

C, basal ray of a quadriradiate with crystals on the lateral surfaces and, towards the tip, on the top surface.

D and E, curved monaxons with crystals on the o.a.-parallel surfaces. Compare with fig. 2, G and H.

F and G, slender monaxons with rhombohedral crystals on their top surfaces.

H and J, triradiates corroded by immersion in 5 per cent. potassium nitrate solution. Note the double nature of the oscular rays caused by the removal of calcite at the o.a.-parallel surfaces.

K, the same for a curved monaxon.

L, part of a longitudinal section of the wall of an oscular tube. The aberrant spicule depicted has the tip of its gastral ray directed at right angles to the optic axis (indicated by the broken line) and against the internal water current (arrow), showing that the latter is not the main cause of the curvature of the normal gastral rays, two of which can also be seen.

M, mounted quadriradiate with crystals on the lateral surface of the gastral ray, and lateral and gastral surfaces of the basal ray. Compare with fig. 1, E.

In each photomicrograph the line represents $50\ \mu$.

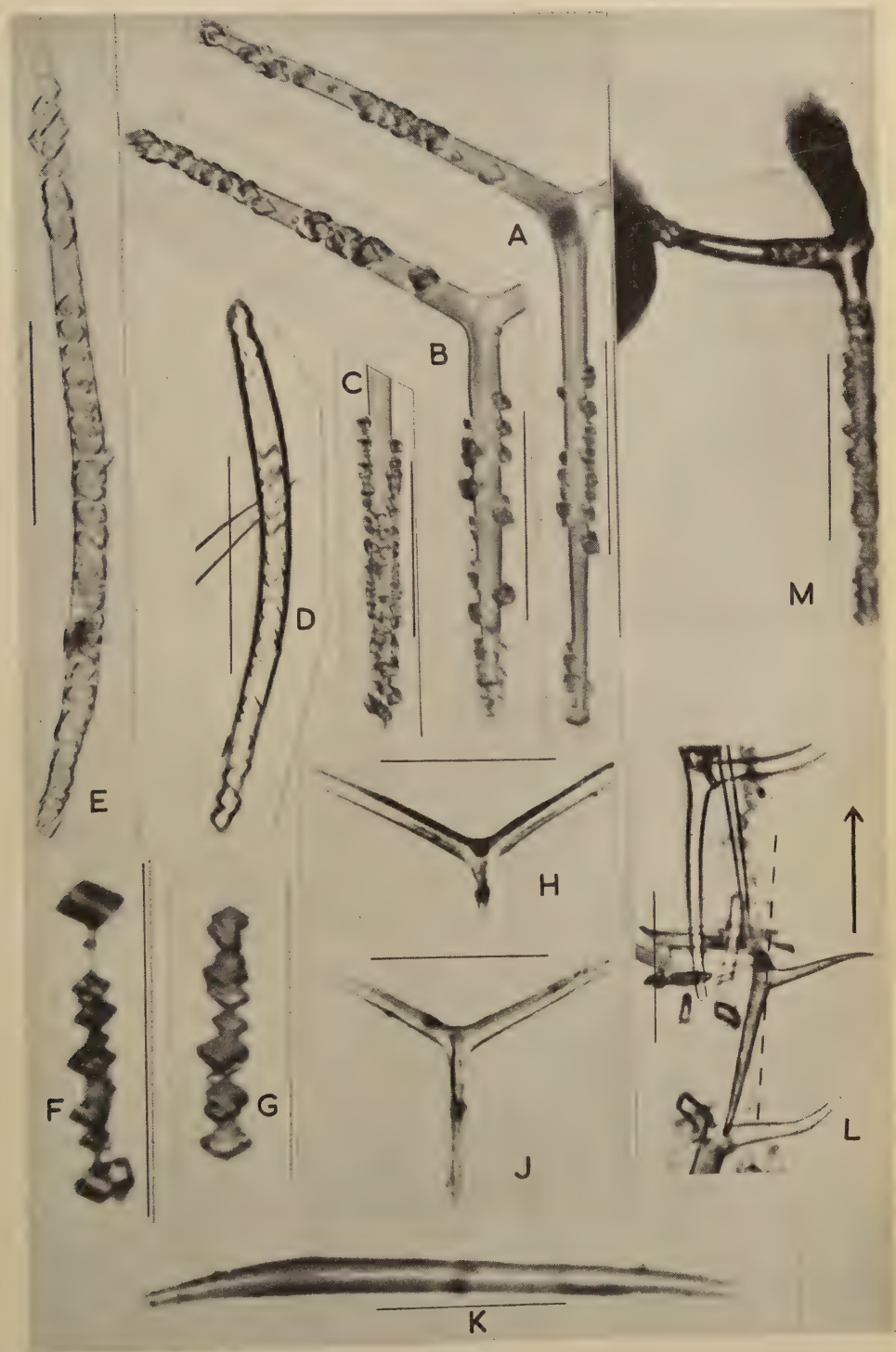


FIG. 4
W. C. JONES

on the gastral and dermal surfaces of the basal rays after the longer period of corrosion, the crystals extending towards the spicule junction from the tip, the lateral crystals, however, leading (fig. 4, c). The oscular rays also acquire crystals on their o.a.-transverse surfaces after greater periods of prior corrosion, and thus once again the formation and spread of the crystals is facilitated by the corrosion, but here the corrosion is acting first on a different part of the spicule surface.

There can be no doubt that the o.a.-parallel surfaces of the spicules are corroded preferentially in the nitrate solution; for, if a slide of spicules is left in this solution for about 8 hours (20° C.), the corroded oscular rays appear as double rods (fig. 4, H, J, and K), both in a plane inclined to the basal

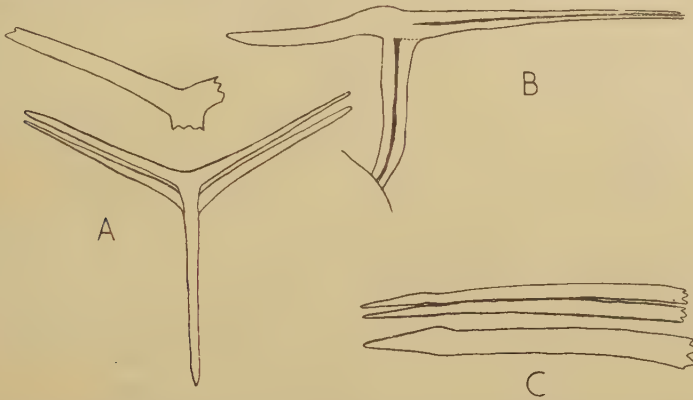


FIG. 5. Camera lucida drawings of *L. complicata* spicules corroded in potassium nitrate solution. The calcite has been dissolved at the o.a.-parallel surfaces (compare with the uncorroded rays drawn alongside). A, triradiate. B, mounted quadriradiate set for side view examination. C, Lance-headed monaxon.

ray, and the same double structure is shown by the gastral and basal rays when the spicule is mounted for side-view examination (fig. 5, B). Furthermore, comparison with the original uncorroded rays indicates very little corrosion of the o.a.-transverse surfaces (fig. 5). Maas (1910) has noted this type of corrosion already with sponges placed in calcium-free sea-water, and it also occurs when solutions of potassium or sodium chloride are employed. These solutions are all nearly neutral and have no chemical action on the spicules, whereas the corrosion on the o.a.-transverse surfaces takes place in acidic or alkaline conditions in which both the carbonic acid and the strong potash solutions respectively used react with the calcite. It would seem therefore that with the more rapid corrosion the faces of the calcite at the o.a.-transverse surfaces of the spicules are selectively attacked and will enlarge as the calcite dissolves away, thereby improving the accessibility of the site for crystal initiation, while with the slower corrosion a different zone of faces (on the o.a.-parallel surfaces) is corroded. It does not seem likely that there is a selective action on the organic sheath; and the inorganic artifact is not produced by the potassium nitrate solution.

The form of the crystals in relation to the spicule rays

The form of the crystals in perfect examples is that of the simple unit rhombohedron of calcite (Miller-Bravais notation: $\{10\bar{1}1\}$) (figs. 2, A; 3, A), and, as Sollas (1886) observed, they possess the same orientation of the optic axis as does the spicule calcite, for the whole darkens simultaneously when

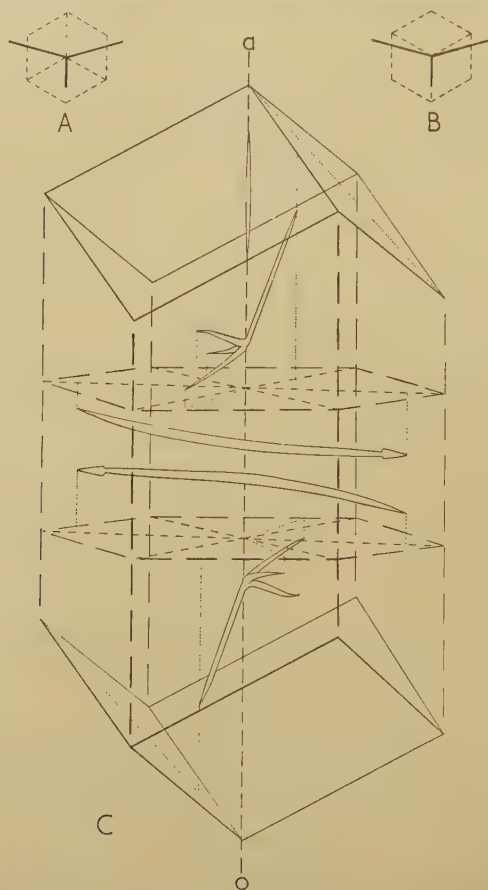


FIG. 6. The crystallographic orientation of spicules of *L. complicata*. A and B, the relationship between the spicule rays and the faces on top of the calcite rhombohedra seen when a triradiate is set with its optic axis coincident with the line of sight and the gastral (A) and the dermal (B) surfaces of the basal ray respectively nearer the eye. Compare with fig. 3. C, diagram indicating the parts of a calcite crystal utilized, as it were, in the formation of the three types of spicules. Each drawing of the quadriradiate and the curved monaxon represents one of three possible settings, since the crystal has a trigonal axis of symmetry. The areas outlined by the thick lines are rhombohedral faces; those parallel to the optic axis and bounded by alternating broken and unbroken lines are prismatic faces. o.a. = optic axis.

mounted in neutral mountant and rotated between crossed nicols. The crystal faces, furthermore, always bear the same relationship to the spicule rays regardless of the position of the crystals on the spicule surface, even though very many spicules derived from three separate specimens (one collected at

Bangor, the other two at Plymouth) have been tested. The relationship is illustrated in fig. 6. When a tri- or quadriradiate is set with the optic axis coincident with the line of sight, the basal ray lies parallel to the edge between the rear two of the three faces on the top of each crystal, provided the dermal surface is uppermost. Inversely, when the gastral surface is nearer the eye, the basal ray lies parallel to the bisector of the rear face (fig. 6, A and B). By imagining the enlargement of a crystal at the ray junction, the diagram in fig. 6, C can be constructed. From the triradiate symmetry of the crystal it is obvious that there will be three equivalent settings for the spicule with the dermal surface uppermost (one shown) and three corresponding settings with the spicule inverted (one shown). The curved monaxons likewise exhibit a constant orientation of their crystal faces, allowing two sets of three arrangements within the giant crystal, one of each type being shown. The slender monaxons are coincident with the optic axis and need not be considered here. Their crystals have the same rhombohedral form, though often certain faces are developed more than others and the crystals have a flattened appearance (figs. 2, E; 4, F and G).

In the diagram (fig. 6, C) the two sets of three rhombohedral faces have been separated to clarify the arrangement of the spicules within. The separated edges of these faces together with the broken lines connecting the separated corners together bound prismatic faces, which von Ebner recognized in the corrosion patterns formed on the lateral surfaces of his acid-treated spicules. Such faces are not developed by the crystals with the crystallizing solution employed, though irregularly shaped crystals are sometimes obtained (particularly when the spicules lie well beneath the surface of the solution) on which faces other than rhombohedral may be exhibited.

When the crystallization has not proceeded sufficiently far, the crystals are small and appear as three-sided pyramids on the spicule surface. The outline of the base is triangular, the relative lengths of the sides depending on the position of the crystal on the ray, but the apex always pointing towards the tip of the basal ray on the gastral surface and in the opposite direction on the dermal surface. These pyramids are the corners of the crystal rhombohedra developing on these surfaces.

The diagram in fig. 6, C is somewhat similar to the one given by von Ebner (1887, fig. 35), derived from corrosion experiments, but closer agreement would be obtained if the giant crystal of one diagram were rotated through 60° about the optic axis without disturbing the enclosed spicules. The triradiate depicted by von Ebner is a primary sagittal spicule, but crystallization experiments with the perregular triradiates of *Clathrina* (= *Leucosolenia*) *coriacea* have given results similar to those obtained with *L. complicata* spicules; the three rays of the former lie in the same crystallographic planes as the basal ray of the latter. Now von Ebner deduced that his giant crystal was a combination of a prism and a negative rhombohedron, the latter being negative because the corrosion triangles formed on the dermal or gastral surfaces of the rays reversed direction soon after corrosion had begun, just

as did the triangles produced similarly on calcspar, with which the persistent orientation corresponds to the negative rhombohedron. The difference between the two diagrams might therefore be due to the development of positive instead of negative rhombohedra in the crystallization experiments; but it is also possible that the calcareous spicules fall into two types, the one having the crystallographic axes set at 60° relative to the other when the spicules are similarly oriented. In this case the crystallization and corrosion experiments would both always reveal the same (either positive or negative) rhombohedral faces on all the spicules of a given species.

It should be noted that the horizontal axes drawn in fig. 6, c are similar to those used by von Ebner, but are not those employed in the Bravais system. In the latter the axes join the mid-points of the oppositely paired edges which zigzag round the middle of the rhombohedron. With the modern system the rhombohedral faces would have the form $\{10\bar{1}1\}$ or for the negative case $\{01\bar{1}1\}$, while the prismatic faces would be $\{11\bar{2}0\}$. The older system has been retained in the diagram to assist comparison with von Ebner's figure, and because it cannot be decided whether the crystallized rhombohedra are positive or negative. If calcite had perfect hexagonal symmetry this would not matter. Because it belongs to the trigonal class there must be some difference (revealed by corrosion markings) between the positive and negative faces, though this difference cannot be detected in the arrangement of the calcium and carbonate ions found as a result of X-ray studies by Bragg (1914).

DISCUSSION

The crystalline structure of the spicules

The crystallization experiments described above have indicated the presence of at least two zones of faces, presumably rhombohedral (on o.a.-transverse surfaces) and prismatic (o.a.-parallel surfaces), which are corroded at relatively different rates in respectively potash and potassium nitrate solutions, while von Ebner has observed these faces enlarged by acidic corrosion. He also saw a zone of scalenohedral faces etched out in between the rhombohedral and prismatic zones on a basal ray, and he decided that the spicule was a single crystal whose surfaces approximate to the various faces of mineral calcite. There is no doubt that many forms of calcite occur naturally (five rhombohedra with different a:c ratios are known, for example, besides the faces of the basal pinacoid, the scalenohedra, and the prisms), so that a fairly smooth replica of a spicule ray could be manufactured by using a combination of appropriate faces. Such a view of the spicule is contrary to that given by Sollas (1885), who concluded from the production of etch lines on the o.a.-parallel surfaces that each ray was made up of greatly elongated separate rhombohedra, and by Maas (1904), who believed that organic matter interconnected the individual rhombohedra. Bütschli (1907), however, has shown that Maas's concept is erroneous, and since ordinary calcspar produces solution-

bodies after mild corrosion just like the spicules, there can be little doubt that von Ebner's view is correct.

Factors controlling spicule form

It has been pointed out already by the author (Jones, 1954*b*) that in its main outlines the form of the triradiate spicule is the result of triplanar growth on the choanoderm surface, the three planes intersecting in the direction of the optic axis, and each ray having a course corresponding to the intersection of its plane of growth with the curvature of the choanoderm. The angles between the three planes are not all 120° for *L. complicata* spicules, however, that between the oscular ray planes (the planar angle) varying from 160° to 140° with the position in the oscular tube. An explanation of the triradiate form must therefore be concerned with (1) the uniplanar growth of each ray, and (2) the planar angle, besides accounting for (3) the orientation of the formative cell complex, (4) the orientation of the optic axis, and (5) the directing influence of the choanoderm. Other problems of course arise in connexion with the association of the formative cells and their secretory activity, but no attempt can be made to explain these as yet.

The mechanism whereby the cell complex becomes oriented has already been suggested (Jones, 1952; 1954*a*) and experimental evidence in support will be published in due course. The optic axis of the calcite, furthermore, has been shown to coincide with the principal symmetry axis of the cell complex, and this has been explained by a process of oriented overgrowth of the calcite upon the organic precursor rodlets (Jones, 1954*a*). It is clear that with the triradiates of *Clathrina* (= *Leucosolenia*) *coriacea*, the calcite could crystallize independently on each of the three rodlets and still fuse up centrally into a single crystalline mass, because both the precursor and the calcite crystal exhibit a triradiately symmetrical arrangement. With *L. complicata* triradiates, however, the planar angle is greater than 120° and, if oriented overgrowth occurs on all three rodlets, the oscular pair cannot be merely splayed out by the forward tilting of the sextet (Jones, 1954*b*), but must themselves be crystallographically uniform with the basal ray precursor, in which case the rodlets would not all have an equivalent internal orientation of their fine structure. Possibly, however, the rodlets maintain a triradiate arrangement when they are very small, enabling oriented overgrowth to occur all over, while later the calcite only crystallizes on preformed calcite.

The curvature of the choanoderm is presumably a factor because the founder cells maintain contact with this layer, perhaps being prevented from leaving it by the relatively firm outer mesogloea (Jones, 1952). The influence of the sponge wall on spicule form has been noted before. Thus Woodland (1905*a*) states that '(larval) spicules . . . which develop in a mass of sponge jelly and which are not in close proximity to two parallel surfaces, are . . . irregular in form' (p. 271) and similarly for *Alcyonium digitatum* spicules (1905*b*). Sollas (1888) suggested that spicules grow along lines of least resistance, while Maas (1900) observed that the curvature of the oscular rays follows that of

the sponge tube and is correspondingly greater in young larvae than in older stages. In fact, on this account, he believed the spicules were flexible, but this seems unlikely in view of their mineral content, and in any case isolated spicules are rigid and spring about when touched with a needle. Probably the earlier-formed spicules are pushed to the outside and then shed when their curvature does not fit that of the tube (Jones, 1954*b*).

Uniplanar growth of the rays and their elliptical cross-section

The experiments described in this paper have indicated a difference in the corrosion properties of the rhombohedral and prismatic faces. The former are more rapidly corroded by potash solution, the latter more rapidly in the nitrate solution. It seems plausible to suppose that these faces, having a different stability in the two solutions, may also crystallize at different rates in certain media. Naturally occurring calcspar crystals often have a form elongated in the direction of the optic axis, indicating that certain conditions favour crystallization on the rhombohedral faces. If such conditions were to exist around the developing spicule rays, one could explain the elliptical shape in cross-section of the oscular and gastral rays, and of curved monaxons, and also the tendency for uniplanar growth. For if the apical calcoblast were freely enclosing the ray tip the ray would become elliptical in cross-section by the ions preferentially crystallizing on the o.a.-transverse surfaces, and growth would be directed at right angles to the optic axis (fig. 7, A). Should the cell be pushed over to one side, as through obstruction by the choanoderm, then less ions would be able to crystallize on the surface against which the (presumed) vacuolar wall is resting; the ray would hence tend to grow towards the free o.a.-transverse surface as the cell moved along in pace with the growth (fig. 7, B), and the curvature would lie in a plane containing the optic axis since the surfaces for the crystallization are transverse to this plane. The apical calcoblast (or the vacuole) would presumably be guided by the uniplanar growth since it is gripping the formed ray some distance behind the tip and would tend to be aligned with it by surface tension.

The existence of a vacuolar wall has not been conclusively demonstrated, but Minchin (1908) refers to 'granules . . . in a distinct vacuole' (p. 350, caption to figs. 19, 20), to a 'sextett with small triradiate, lying in a clear space limited by a distinct sheath' (fig. 23), to 'the frequent presence in the founder cell of a distinct, clear space . . . appearing like a mould in which the secretion . . . is laid down' (p. 309), and to the fact that 'the calcite . . . does not at once completely fill this space' (p. 309). On the other hand, he remarks that when the spicule is partly corroded the 'shaft often appears hollow, containing a cavity or canal continuous with the clear space' (p. 309), and since the axial filament was hence not separately distinguished, it is possible that the space is diffusely filled with organic matter and is the precursor itself. However, it seems most likely that the crystallizing solution is contained in a vacuole around the developing ray.

It has been stressed earlier (Jones, 1954*b*) that the growth of the oscular

rays only tends to be uniplanar; quite often the rays curve gradually outwards when seen in planar view. This would be expected from fig. 7, D, for the most accessible parts of the o.a.-transverse surfaces lie towards the pinacoderm side. The uniplanar growth is best seen with the gastral and basal rays and

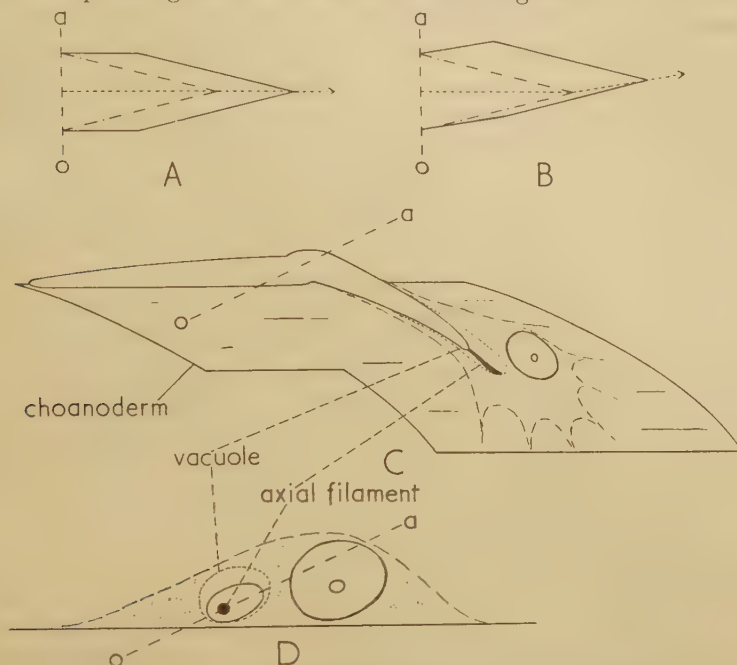


FIG. 7. Diagrammatic explanation of the uniplanar curvature of the rays. A, equal crystallization on the two o.a.-transverse surfaces results in growth at right angles to the optic axis (o.a.). B, unequal crystallization results in a change in the direction of growth in the plane containing the optic axis. C, a triadial with its right oscular ray founder-cell, seen from the right side (diagrammatic). D, a section in an axial plane of the tube passing through the founder cell. The choanoderm displaces the cell and vacuole upwards on the tip, largely preventing the crystallization on the lower o.a.-transverse surface. Growth will tend to be upwards in the plane containing the optic axis, but it will not in this case be strictly uniplanar, since the crystallization on the lower surface is asymmetrically inhibited; the oscular rays would appear splayed out in planar view when fully grown, as is often the case (see fig. 3, c). The vacuole presumably tends to continue the direction of the earlier-formed part of the ray, because the surface tension in the vacuolar wall will tend to orientate it symmetrically on the ray tip.

the curved monaxons; in these the founder cell is dislodged in the plane of the optic axis and hence the hindrance to crystallization on one or other o.a.-transverse surface will be complete.

The basal ray is circular in cross-section, which would be expected from the preferential tendency for crystallization to start in the four centro-lateral rows. This again will give rise to a uniplanar curvature if the founder cell is displaced on the tip.

Curved monaxons

The curved monaxons exhibit the uniplanar curvature well and they are also elliptical in cross-section, the long axis of the ellipse lying in the direction

of the optic axis. Their curvature is thus probably the result of a restraint imposed upon the crystallization on one of the o.a.-transverse surfaces caused by the displacement of the founder calcoblast to the opposite side of the spicule by the choanoderm. This is supported by the form and arrangement of the curved monaxons at different stages of development. The spicule is at first a relatively straight rod between the two formative cells (see Minchin's (1908) drawings, pl. 17, figs. 10 and 11), and begins to curve proximally when the shaft is about $90\ \mu$ long and when the thickener cell (which remains in contact with the pinacoderm) has commenced to move down the rod, i.e. when the lance-head has begun to protrude through the surface layer (fig. 8, A). The spicule then stretches from pinacoderm to choanoderm and is

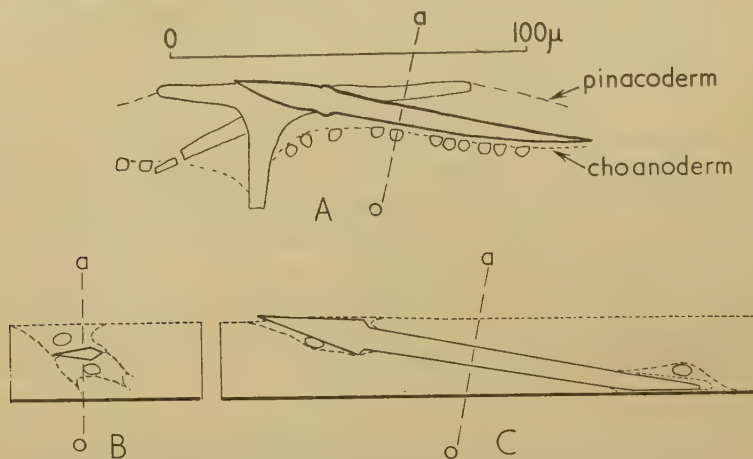


FIG. 8. A, camera lucida drawing of a young curved monaxon of *L. complicata*. The distal region is straight (length about $90\ \mu$). B and C, diagrams derived from the drawings of Minchin (1908) indicating two early stages in the development of the curved monaxon. It is believed that the pinacoderm shears forwards relative to the choanoderm, thereby directing the inclination of the spicule rudiment in between the two layers. Growth initially is perpendicular to the optic axis, but the protrusion of the tip through the pinacoderm prevents any further change in the inclination of the spicule to this layer (which would accommodate the growth increase) and the founder-cell is hence forced over to the outer side of the growing tip by the choanoderm. The crystallization on the outer o.a.-transverse surface then produces the curvature in the plane containing the optic axis (o.a.).

prevented from changing its inclination to the pinacoderm (which would accommodate the growth increase) because the tension in this layer will prevent further rotation once the spicule tip has protruded. The choanoderm must then deflect the founder cell towards the pinacoderm side (fig. 8, C) and since the optic axis lies in an axial plane of the oscular tube, crystallization takes place mainly towards this side and the ray becomes curved in the axial plane. Why the optic axis should lie in this plane and why the founder cell should initially move in the basal direction need explanation, but it would appear that the mother-cell divides first, cutting off a daughter-cell on the choanoderm side and thereby setting the long axis of the cell pair (and hence the spicule optic axis; Jones, 1954a) in the axial plane (fig. 8, B), and that the

pinacoderm shifts forwards (towards the osculum) relative to the underlying choanoderm to which the inner cell is anchored by its cell processes, much as the mesogloea is believed to move (Jones, 1952). Whatever the details of this mechanism, the founder cell always lies on the concave side of the curvature (see Minchin, 1908, pl. 17, figs. 12-15; pl. 20, figs. 80-83).

Slender monaxons

The optic axis of the slender monaxons coincides with the morphological axis and the tendency for the calcite to crystallize in the direction of the former is thus very evident. It is also likely that the rapid growth of these spicules (Maas, 1900) is due to the ease with which the crystallization occurs at the o.a.-transverse surfaces.

The pattern of orientation of the slender monaxons (Jones, 1954a), like the development of the curved monaxons, suggests that the pinacoderm shears forwards relative to the choanoderm as the oscular tube grows. This is only true, however, for the distal parts; for towards the base the orientation is roughly perpendicular to the tube surface. Thus one would expect that the curved monaxons are only formed in the distal region, while the slender monaxons, which are very readily shed, are formed all over the tube. However, no attempt has yet been made to verify this. The slender monaxons are inclined to the pinacoderm before being protruded through the surface (Minchin, 1908, pl. 18, figs. 42 and 44), so that the orientation cannot be caused by the water currents set up around the free end of the tube by the exhalant jet.

The basal ray of the triradiates is somewhat similar to the slender monaxon in having the optic axis nearly coincident with the morphological axis. It is thus possible that the precocious development of the basal ray in *L. complicata* (Minchin, 1908) is due to the easier crystallization in roughly the direction of the optic axis. However, this is not the sole factor, as the author has already pointed out (Jones, 1954a).

Gastral rays

The shape of the gastral rays of the quadriradiates and its dependence on the position in the oscular tube have previously been described (Jones, 1954b). Apart from those at the oscular edge, the gastral rays have three parts, a short proximal portion roughly perpendicular to the optic axis, a central region perpendicular to the choanoderm, and a curved distal region once more perpendicular to the optic axis. In cross-section the rays are elliptical with the long axis of the ellipse, like the curvature and the optic axis, lying in an axial plane of the oscular tube. Thus once again the uniplanar growth is probably the result of preferential crystallization mainly on the two o.a.-transverse surfaces: when the formative cell is perched on the nearly fully grown tip it is unrestricted and the growth is perpendicular to the optic axis; when close to the choanoderm the cell moves in the direction perpendicular to this layer and the crystallization on the two surfaces becomes unequal. What causes

this movement perpendicular to the choanoderm is at present unknown. It does not seem to be correlated with the activity of the flagella, which drive the water directly inwards, since the central region is present in gastral rays occurring above the choanocyte zone (Jones, 1954*b*, fig. 5, F), although possibly the position of the choanocyte limit has receded since these rays were formed.

At the oscular edge the division of the gastral rays into three distinct regions is absent and the ray curves into a direction parallel to the internal water current. In this region the cross-sectional area of the tube cavity is less than that over most of the length and furthermore there are no choanocytes to produce an inwardly directed stream. In consequence the outflowing jet is brought to bear on the gastral ray during the whole of its development beyond the inner surface of the wall, and it seems plausible that the formative calcolblast should thereby be deflected on to the leeward side so that crystallization occurs most easily there, producing the uniplanar curvature. Bidder (1898) has already noted that the formative cell lies on the concave side of the gastral ray, and the deflecting action of the internal water current has also been considered by Sollas (1877), who explained the inclination of the spines projecting into the spongocoel of the siliceous sponge, *Stauro-nema*, as the result of two pressures applied at right angles, one caused by the water flow and the other arising from the tendency of the spine to grow inwards perpendicularly to the surface. However, Minchin (1908) opposed a similar assertion of Woodland (1905*a*) by stating that in the non-oscular diverticula of *L. lieberkühnii* the gastral rays forming distally, just before the tube opens, point against the presumed direction of water flow. Moreover, the aberrant spicule depicted in fig. 4, L has its gastral ray directed against the water current, but at right angles to the optic axis, and other similar aberrants have also been observed by the author. It thus appears that the water current only modifies the distal growth direction, the extent varying with the position in the tube; the major tendency is for growth at right angles to the optic axis.

Thus the elliptical cross-section of the rays (basal ray and slender monaxons excepted), their tendency to grow perpendicular to the optic axis when growth is unimpeded, their curvature in a plane containing the optic axis, and the relative rapidity of growth of the slender monaxons, are all largely explicable in terms of the crystallomorphic properties of the crystallizing medium.

The planar angle

The explanation for the tendency for uniplanar growth requires an initially directed ray primordium to start the process off, and the disposition of the primordia must now be explained. It is clear from a comparison of the shapes of young triradiates (Jones, 1954*b*, fig. 1) that the course taken by the oscular ray founder-cells is variable even in the earliest stages of spicule formation and depends on the position in the tube. The planar angle also varies continuously (from 160° to 140°) according to the position and is not directly correlated with the optic angle. It hence cannot be attributed solely to some

crystallizing property of the material since it is highly improbable that the direction of crystallization could be so diversely controlled by the composition of the medium. Furthermore, it cannot be explained by a splaying out of the precursor rodlets as the sextet tilts forwards and the oscular founder cells are squashed (Jones, 1954a), since, where the tilting is least (at the oscular edge), there the planar angle is greatest.

The planar angle hence affords a problem. It cannot be controlled by the initial distribution of the cells in the formative sextet, for aberrants like that shown in fig. 1, c have oscular rays parallel to one another and yet obviously the two apical calcoblasts must have been widely separated when they set out

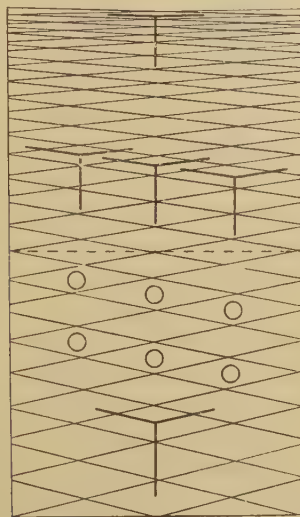


FIG. 9. The spiral organization of the oscular tube of *L. complicata* shown diagrammatically. The spicules lie on spirals wound round the tube axis, the spirals opening out towards the base of the tube. The pores also tend to lie on spirals. Note the relation between the interlacing spirals and the oscular rays of the T-spicule at the oscular edge, and of the Y-spicule at the base of the tube. Explanation in the text.

on their parallel courses. Having set out, the uniplanar growth of each ray and the curvature of the choanoderm would together make the rays parallel; but what has directed the cells initially? It would appear that some guidance is being given by the wall of the sponge, and a clue is afforded by the arrangement of the spicules and pores in clockwise and anticlockwise spirals round the tube axis. Maas (1900) has already drawn attention to this spiralling in a young syconoid sponge. In *L. complicata* there is a similar tendency, the spirals being, however, tightly wound at the oscular edge and loosening out towards the base of the tube (fig. 9). Such an opening out could be a consequence of longitudinal growth.

The pores (and probably their precursor cells in the porocyte epithelium) are also arranged in spirals and, like the spicules, spread apart longitudinally as the tube grows (Jones, 1952). A relationship thus seems to exist between

the porocytes and the spicules and this is supported by the fact that in the young olynthus each spicule lies in front of a pore (Minchin, 1898, p. 546). Also the calcoblasts which produce the gastral rays arise from a neighbouring porocyte (Minchin, 1898). The relationship, however, is less clear in older tubes because the old pores close (Prenant, 1925; Jones, 1952) and new pores and spicules develop all over the surface.

Now evidence has been obtained (to be published) that the pores are connected together and to the porocyte epithelium, and probably form a continuous membrane on which the choanocytes are settled and to the outside of which the sextets are attached (Jones, 1952). The spiral arrangement of the porocytes forming this layer is possibly associated with the presence of similarly spiralling, sub-microscopic fibrils or micellae in the membrane, and it is conceivable that the oscular ray founder-cells are set by these fibrils, much as the tip of a nerve axon is guided (Weiss, 1945). Within the founder cells the precursor rodlets will crystallize in the direction of least mechanical resistance and will thus be inclined to one another by the same angle as lies between the interlacing spirals at the site. Thus T-spicules will be formed at the oscular edge and Y-spicules towards the base of the tube, as is the case (fig. 9). Furthermore, since the optic axis is inclined to the plane of the three rodlets, the planar angle will have a value in between the surface view angle and that seen in transverse view (180° nearly). The former angle is greatest at the oscular edge and hence there the planar angle will also be greatest for the same inclination of the optic axis, and this also is the case. Thus the presence of a spiralling micellar organization corresponding to the arrangement of the spicules and pores would explain the dependence of the planar angle and the shape of the triradiates in surface view on their position in the tube. Whether the founder cells are guided by the same micellar fibrils throughout the growth of the ray, or allow the process of uniplanar crystallization to take complete control cannot be decided for the present.

The basal ray founder cell is set symmetrically behind the oscular pair so that the growth of the ray is parallel to the length of the tube. Its direction points to the tube base because of the previous orientation of the sextet by the process of mesogloal shear (Jones, 1952; 1954a).

Sollas (1888) has already suggested that spicules grow along lines of least resistance. He pointed out that a pellicle which fissures under tension forms cracks that are rectilinear or inclined at angles of 60° or 120° to each other, the hexagonal arrangement being the most likely since the perimeter is then smallest in relation to the enclosed area. Such fissures are not produced in the sponge tissue, but nevertheless Sollas believed that lines of least resistance occur where they would tend to arise, and he claimed that the simplest calcareous spicules are shaped by the growth of the rays along these lines. However, the alate form of the spicules of *L. complicata* is contrary to this view, while none of the triradiates is oriented in the reverse direction as one would expect were the form governed solely by hexagonal patterns of lines of least resistance.

It thus appears that the spicule form is caused by the interaction of extrinsic and intrinsic factors, the latter depending upon the properties of the crystallizing material. The extrinsic factors, for example the number of founder-cells (controlling the number of sites of crystallization), the mesogloecal shear (setting the sextet in the required orientation), the choanoderm curvature, and the presumed sub-microscopic fibrillar system, are features of the general organization of the oscular tube and presumably under the control of the gene complex.

The crystallization experiments were begun at the Department of Zoology, Cambridge, and I wish to express my thanks to Professor Sir James Gray, F.R.S., and the staff for their help. I am also greatly indebted to Professor F. W. Rogers Brambell, F.R.S., for affording me the facilities and time to continue the experiments, and to the staff of the Department of Zoology, U.C.N.W., Bangor.

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The Function and Metabolism of Certain Insect Muscles in Relation to their Structure

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With 4 plates (figs. 2, 3, 6, and 7)

SUMMARY

Electron microscopic observations on ultrathin sections of the red thoracic flight-muscles and white leg muscles of *Hydrophilus* and *Dytiscus* are reported.

In red muscle-fibres with high values in frequency of contraction, oxygen consumption, and dehydrogenase activity, the single fibrils are completely surrounded by huge mitochondria. Tracheoles penetrate the sarcolemma and supply the mitochondria with oxygen by intracellular branches. In the less active white muscle fibres, mitochondria are found irregularly scattered between the fibrils or along the I band. The intracellular tracheolization is sparse but an endoplasmic reticulum is widely spread between the synfibrillar contractile material. The same muscles of the two insects differ considerably in detail.

INTRODUCTION

THE highly specialized functions of insect muscles make these the best models for the study of the relationships between function, metabolism, and structure. This had been noted by the earlier microscopists such as Knoll (1889) and Holmgren (1909, 1913), but in recent years has not received sufficient attention. Marked metabolic differences are to be found among the various muscles of various insects, these differences being closely related to the function of the muscles and the phylogenetic position of the insect involved (see Pérez González and Edwards, 1954, for summary of the literature). Physiological studies of insect flight (Roeder, 1951) have also brought to light considerable differences in nerve-muscle relationships between the higher and lower insects. Structural differences in the tracheation of muscle fibres, differences in colour of fibres, and variations of ultrastructure of isolated fibrils also occur from muscle to muscle in the insect (Edwards and others, 1954a, 1954b). In mammalian muscle (Ruska, 1954) and fowl-breast muscle (Bennett and Porter, 1953), electron microscope studies have suggested that the endoplasmic reticular and mitochondrial systems may be closely related to the process of contraction within the fibril and further related to specialization in muscle function.

With these facts in mind we have begun to study ultrathin sections of certain insect muscles, with the electron microscope, to determine the relationship of metabolism and specialization in function to principles of structure. Particularly we have been interested in the relationship of the tracheoles to the mitochondria, and the roles played by the mitochondria

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and reticular systems in widely differing types of muscular functions, such as flying and walking.

MATERIAL AND METHODS

The muscles used were the dorso-longitudinal, indirect, flight (red), and the coxal levator (white) muscles of adult *Hydrophilus ater* and *Dytiscus* spp. The muscles were observed in both the contracted and stretched states. They

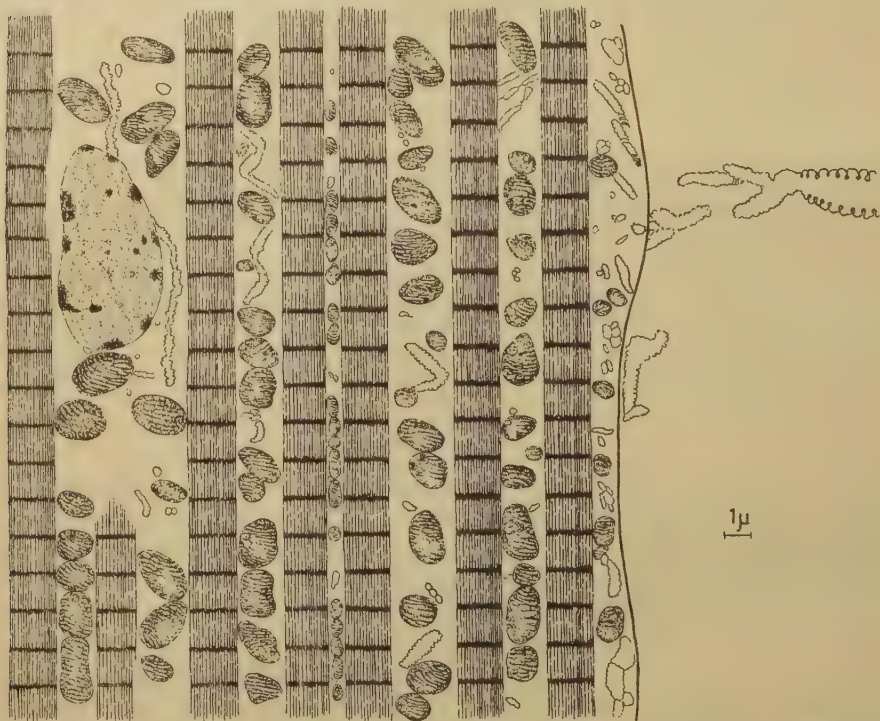
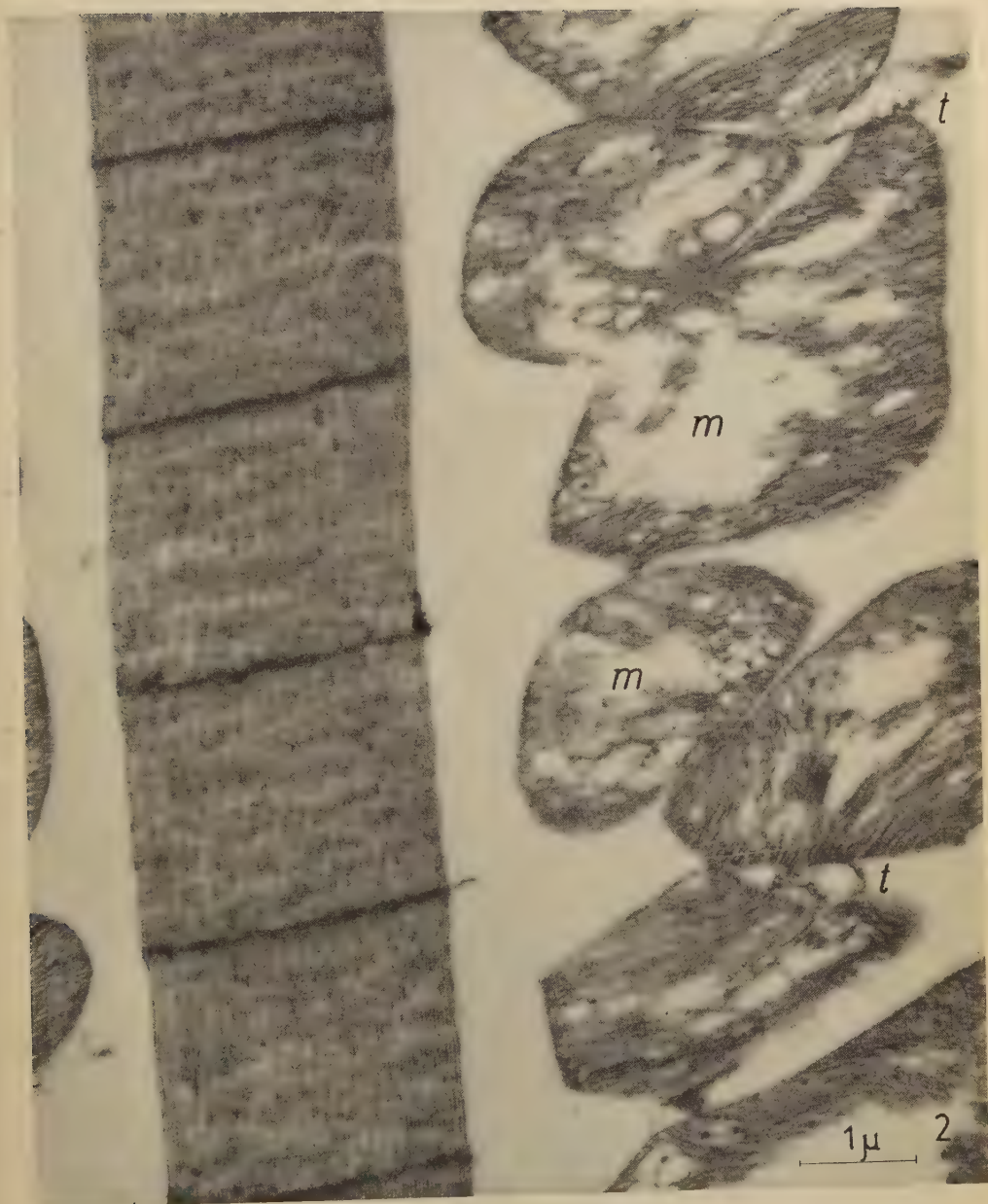


FIG. 1. Red fibre of *Hydrophilus*. Many tracheoles outside and inside the thin sarcolemma, intermingled with the mitochondria between the contracted myofibrils and located close to one nucleus.

were fixed in 1 per cent. buffered osmium tetroxide solution, according to the method of Palade (1952), and embedded in methacrylate by the conventional method for electron microscopy. The sections were cut with a microtome built in the Rockefeller Institute for Medical Research. The microscope used was the Siemens UM-100b, of the Instituto Butantan in São Paulo. From several micrographs, drawings were composed to show the overall construction and to reduce the number of illustrations (see figs. 1, 4, 5, and 8). The originals were exhibited at the International Conference of the Joint Commission on Electron Microscopy, held at London University, 16-21 July 1954.

FIG. 2 (plate). Red fibre of *Hydrophilus*. Electron micrograph. Four contracted fibril segments; *m*, mitochondria; *t*, tracheoles.



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RESULTS

Oxygen supply

The tracheoles have previously been thought to terminate on the surface of the muscle fibre. The red flight-muscle fibres have a large external tracheal supply; the white leg-muscle fibres have few tracheal branches. In our preparations we have seen that the tracheoles actually penetrate all muscle fibres, but the number and distribution of penetrating tracheoles vary with the type of muscle.

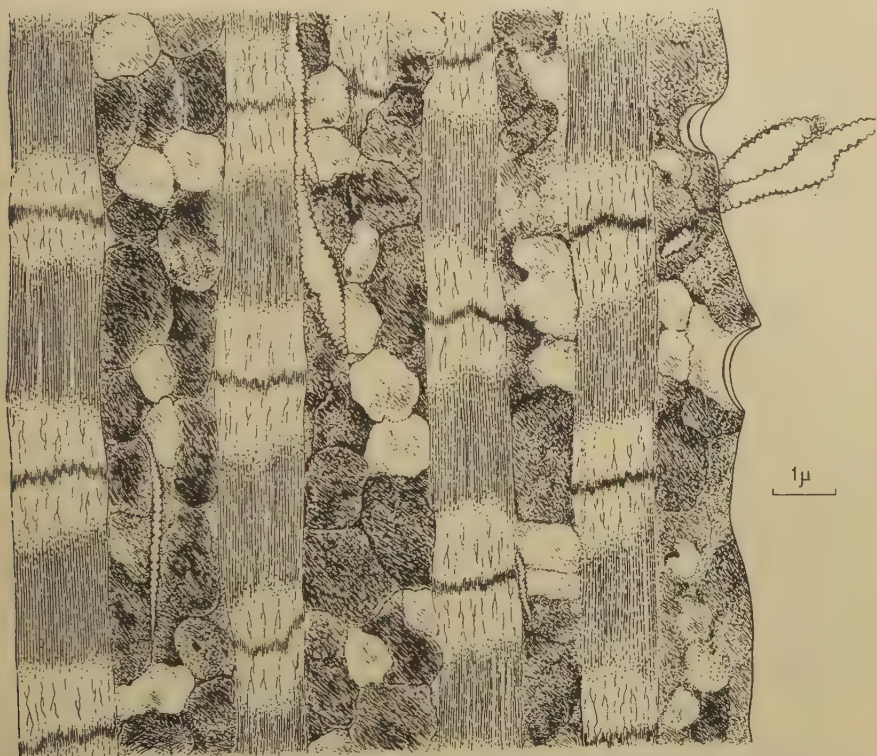


FIG. 4. Red fibre of *Dytiscus*. Few tracheoles outside and inside the thin sarcolemma; they are attached to the closely packed mitochondria between the stretched myofibrils.

In the sections of *Hydrophilus* red fibres (figs. 1 and 2) it can be seen that as the trachea approaches the fibre surface it gives off numerous fine branches (tracheoles) which penetrate the sarcolemma at various points. Just beneath the sarcolemma may be seen many branches in cross, tangential, and longitudinal section. Between the fibrils, and closely associated with the mitochondria, are found cross-sections of very fine, intracellular tracheoles throughout the entire fibre. It is interesting to note that well in the interior

FIG. 3 (plate). Red fibre of *Dytiscus*. Electron micrograph. One stretched fibril segment; *m*, mitochondria; *t*, tracheoles.

of the fibre the tracheoles appear to be uniform in diameter; they are often in clusters, and always near or actually in contact with the mitochondria. The space between the fibrils in this muscle is considerable and appears to be filled principally with the fine tracheoles and large mitochondria.

In the sections of the red flight-muscle of *Dytiscus* (figs. 3 and 4) fewer tracheoles are observed. The general tracheal picture is similar to that of

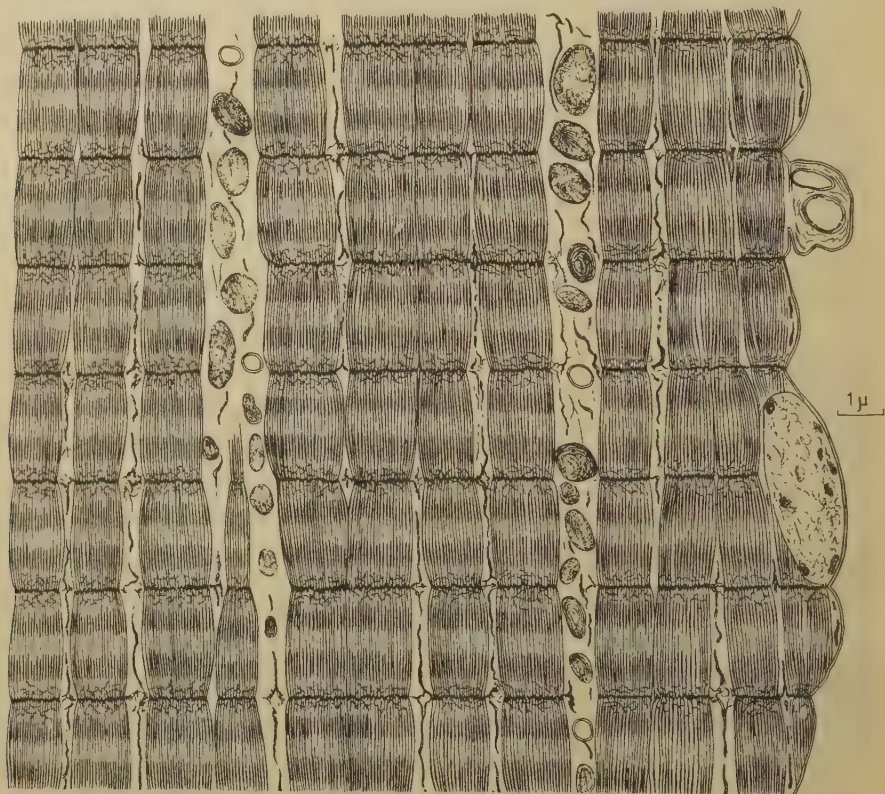


FIG. 5. White fibre of *Hydrophilus*. Few tracheoles in cross-section beneath the sarcolemma and between the mitochondria. The endoplasmic reticulum between the myofibrils spreads along the Z bands, which are attached to the sarcolemma.

Hydrophilus, in the branching of the trachea at the surface and the penetration by the finer tracheoles into the fibre. Differences appear in that there are fewer tracheoles within the fibre and they generally run longitudinally between the fibrils, together with the mitochondria. Interestingly enough the tracheole diameter in this muscle is greater in relation to the fibril diameter than in the *Hydrophilus* muscle. The mitochondria of this muscle are much more closely packed and this may well influence the tracheole distribution. Thus, it appears that in the red flight-muscle fibres of both insects the tracheoles

FIG. 6 (plate). White fibre of *Hydrophilus*. Electron micrograph. Fibrils; *m*, mitochondria; *r*, endoplasmic reticulum.



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and mitochondria form a continuous system in which lie the discontinuous fibrils.

In the sections of the white muscles (figs. 5-8) few tracheoles were observed. They penetrated the sarcolemma, thus entering the fibres, at relatively few

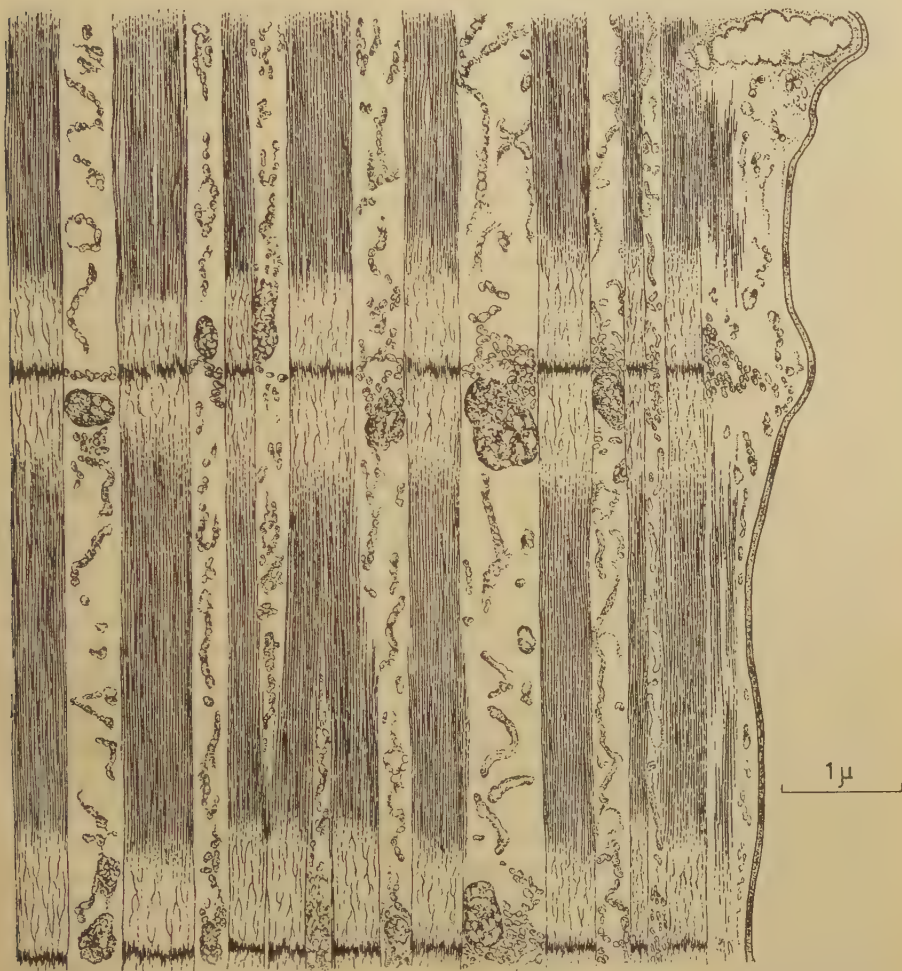


FIG. 8. White fibre of *Dytiscus*. One tracheole beneath the sarcolemma. Endoplasmic reticulum between the myofibrils, accumulating in the I region, where small mitochondria can be seen.

regions along its length. In the white muscle of *Hydrophilus* relatively few cross-sections of tracheoles were seen among the mitochondria, which sometimes occurred in rows between the myofibrils. In the white muscle of *Dytiscus* only subsarcolemmal tracheoles were observed.

FIG. 7 (plate). White fibre of *Dytiscus*. Electron micrograph. Fibrils; *m*, mitochondria, close to the Z bands; *r*, endoplasmic reticulum.

In no muscle were tracheoles seen to enter the myofibrils or to have a definite relation to the muscle bands.

Loci of oxidations

The outstanding characteristic of the red flight-muscle of these two insects is the number and size of the sarcosomes, i.e. the mitochondria of insect muscles.

In the *Hydrophilus* flight-muscle the mitochondria are found in rows parallel to and between the myofibrils and closely associated with the numerous tracheole branches throughout the entire fibre. Just beneath the sarcolemma there are few mitochondria, relatively speaking, scattered among the larger tracheolar branches. Within the body of the fibre, however, internal to the peripheral fibrils, the mitochondria are extremely numerous, one usually touching upon the next, separated from the fibrils in most cases by some distance. In number they average about one mitochondrion to each fibril segment. The *Hydrophilus* mitochondria are largely ovoid or spherical, averaging $2.2 \times 1.5 \mu$. The internal structure of the mitochondria appears like a pile of irregular corrugated cardboard. When cut normal to the planes one sees straight to wavy lines, usually oriented at right angles to the alignment of the myofilaments. This pattern is interrupted by lacunae, sometimes opening to the surface. When cut with the planes, the mitochondria more often present the lacunar picture. It may be possible that the tracheoles in contact with the mitochondria open into the lacunar system.

In the red flight-muscle fibre of *Dytiscus* the mitochondria appear to be more numerous, much more closely packed, of the same size generally (averaging $2.5 \times 1.5 \mu$), and often compressed into polygonal shapes with clearly marked limiting membranes. As in *Hydrophilus* a row of mitochondria is found between the sarcolemma and the peripheral fibrils, and thereafter there are single or double rows between and parallel to the succeeding myofibrils. In number they appear to be about 2–3 per segment, tightly squeezed one against the other. Inasmuch as the tracheoles in this muscle are larger in diameter, fewer in number, and longitudinally arranged between the fibrils, the picture of a mitochondria-tracheole continuous system is less clearly seen here than in the *Hydrophilus* muscle fibre. However, the tracheoles again appear to be more correlated with the mitochondria than with the myofibrils. The internal structure of the mitochondria of the red muscle of *Dytiscus*, although fundamentally similar to that of *Hydrophilus*, is much finer and is visible only in exceptionally thin sections with good resolution.

Characteristic of the *Dytiscus* flight muscle, and giving the sections a checker-board appearance, are numerous spherical bodies of uniform size, slightly smaller than the mitochondria described above and scattered among them. The bodies seem to be more often associated with the I than the A region of the fibril, and occur on the average as one such body to each fibril segment. A definite limiting membrane is visible between any two of these bodies. Internally the structure is similar to that of the other mitochondria,

but shows a looser and thinner arrangement. These bodies can be interpreted as either mitochondria which have undergone a physiological change, or else a different type of mitochondria. We are more inclined to the former view, believing that this could represent a reversible metabolic state or irreversible ageing.

The white coxal muscles characteristically have very few mitochondria and these are of small size. The outstanding characteristic of the white muscles is the endoplasmic reticulum. The *Hydrophilus* white muscle shows great similarity to the mammalian muscle structure shown by Ruska (1954). The mitochondria are aligned in longitudinal rows separating bundles of myofibrils, the fibrils themselves being separated by the endoplasmic reticulum. In *Dytiscus* the mitochondria are arranged singly in transverse rows, i.e. always at the level of the I band between the individual fibrils. In the white muscles of both insects the internal structure of the mitochondria appears to be similar to but less distinct than that of the mitochondria of the red muscle fibres. In form they are roughly oval, averaging $0.4 \times 1.0 \mu$.

Endoplasmic reticulum

The endoplasmic reticulum is peculiar to the white muscles but apparently lacking or much less developed in the flight-muscle fibres. It appears to be a continuous system located between the myofibrils and connected to them at the level of the I region. Thus in low magnifications a longitudinal section through a white fibre shows an apparently continuous Z line. On amplification, however, one can see that the Z is actually restricted to the myofibril but that connexion of one fibril to the next is made by the reticular system. In *Hydrophilus* the system appears to be represented by dark threads between the fibrils, sending very fine branches between the myofilaments predominantly at the level of the I region. In *Dytiscus*, on the other hand, the system is definitely tubular and appears rather to surround the fibrils than to enter them. The greatest concentration of tubes is at the Z line, but a secondary accumulation occurs near the middle of each segment. In the red muscle of *Hydrophilus* faint traces of endoplasmic material may be seen in the vicinity of Z.

Myofibrils

The sections of the fibrils confirm the older findings concerning the differences between red and white insect muscles. The red fibrils in *Hydrophilus* are more uniform in diameter and are more clearly separated than the white fibrils. Actually in the white muscle fibres the fibrils form a synfibrillar continuous system. In all fibrils of both insects the myofilaments are clearly visible; they are continuous, at least in the contracted state, throughout both A and I regions. The filaments of the white muscle of *Hydrophilus* appear to be more loosely packed and in less orderly arrangement than in the red and the white muscles of *Dytiscus*. In both types of muscle it could be seen that the fibrils are certainly composed of more than just filaments.

Further details of their structure will be discussed elsewhere. It should be noted here, however, that only in the *Hydrophilus* white muscle was the Z line seen to be attached to the sarcolemma, giving it a scalloped form.

DISCUSSION

The results have shown very clearly that the specialized functions of the muscles studied are based upon specialization in structure. The flight-muscle, with its high velocity of movement and its high oxygen consumption, possesses an intracellular tracheole system closely linked to large numbers of huge mitochondria, thus providing the carriers of the oxidative enzymes with an ample oxygen supply. The flight-muscle is therefore essentially a fast-acting energy-producing machine. The oxidative mechanism is very close to the contractile mechanism. The loci of oxidations completely surround each fibril. This is a logical mechanism inasmuch as the flight-muscle fibril must have energy available to it, must have end products removed, and must get back resynthesized carbohydrates and phosphates as rapidly as possible for repetitive contractions. To this end strength has been sacrificed, most of the fibre space being occupied by the mitochondria-tracheole system.

The white muscle is slower, capable of continuous tension, has a lower oxygen consumption, but needs a continuous low energy supply. The greater part of the fibrillar areas is filled with the contractile substance, the mitochondria occupying a very small fraction of the space. Actually this muscle has less mitochondria and tracheolization than would be expected on the basis of its metabolic activity, but we must keep in mind that the central part of the white *Dytiscus* muscle contains more cytoplasmic material. The remaining space between the fibrils is occupied by the endoplasmic reticulum. The white muscles apparently are constructed for strength rather than fast action and have therefore sacrificed oxidative capacity.

The presence of the endoplasmic reticulum in the white muscle and the mitochondria-tracheole system in the red muscle raises the question of their respective roles in the metabolism and functions of these muscles. The mitochondrial system is predominant in mammalian diaphragm (Ruska, 1954), in vertebrate heart-muscle (Kisch and Philpott, 1953), and insect flight-muscle. The endoplasmic reticulum has been found to occur in the breast muscle of the hen (Bennett and Porter, 1953), mammalian leg muscles (Ruska, 1954), and in insect white muscles. Thus the mitochondrial system appears to be linked to the need for repeated bursts of energy for fast and strong action, whereas the basophil reticular system is more suitable for the maintenance of tension where more time is available for resynthesis.

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A Histochemical Study of the Cytoplasmic Inclusions of the Epithelial Cells in the Epididymis of the Mouse

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SUMMARY

1. A histochemical study has been made of the cytoplasmic inclusions of the epithelial cells lining the storage section of the canal in the head of the epididymis of the mouse.

2. Spherical bodies, usually situated in the vicinity of the nucleus and here referred to as *juxta-nuclear bodies*, are shown to consist of cerebroside with some phospholipid. The *juxta-nuclear bodies* have been regarded by other authors as derived from the nucleolus, but there is no histochemical similarity.

3. A large *supra-nuclear body* exists, consisting of a matrix of protein and carbohydrate with enmeshed spheres. The latter do not colour with any dye or histochemical reagent used in this investigation. They are partially or completely surrounded by rims or crescents consisting wholly or mainly of lipid.

4. *Droplets* and much smaller *granules* are present in the region between the supra-nuclear body and the free border of the cell. The contents of the former show no histochemical evidence of the presence of organic matter, while the latter consist of protein and carbohydrate.

INTRODUCTION

THE epithelial cells of the head of the epididymis of the mouse have recently been subjected to careful study by the electron-microscope (Dalton and Felix, 1954). The present investigation was undertaken in the belief that such studies might lead to error unless controlled by very full use of the light-microscope on the same material. In particular, the work with the electron-microscope has been done mainly with a single fixative, osmium tetroxide, which is capable of misleading through the deposition of osmium dioxide, which scatters electrons; and in its ordinary usage the electron-microscope gives no chemical information. Some of the objects in the cytoplasm of the epithelial cells of the head of the epididymis of the mouse are quite large enough for morphological and chemical studies with the light-microscope.

Good morphological descriptions of the cell in question have been published by Nassonov (1924), Ludford (1925), and Benoit (1926).

In the present studies their findings have been carefully checked. It was felt that there was a considerable gap in knowledge, partly because there has been too much reliance in the past on the study of deposited particles of osmium dioxide or of silver, and partly because scarcely any histochemical findings have been reported.

As Benoit (1926) showed, the head of the epididymis of the mouse contains tubules of two kinds: (1) the initial segment, in which spermatozoa are not stored, and (2) the storage region. Everything in the present paper refers to the latter region except where the contrary is distinctly stated.

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In the present paper the end of the cell next the lumen of the tubule will be called the apical or upper end; the other end, the basal or lower.

MATERIAL AND METHODS

Pieces of fresh epididymis of the common house-mouse were placed in a number of fixatives (referred to later) within 1 to 2 minutes of killing the animal. After fixation overnight and thorough washing in running water next day, both paraffin and gelatine sections were prepared and examined unstained or stained by various techniques. In addition to routine histological staining methods (Ehrlich's haematoxylin and eosin, Heidenhain's iron haematoxylin, toluidine blue, phloxine-tartrazine (Lendrum, 1947)), Golgi techniques (of Kolatchev, Mann-Kopsch, and Aoyama), and Metzner's modification of Altmann's stain for mitochondria, the following histochemical techniques were applied to appropriately fixed and embedded tissue:

(1) Sudan black B on gelatine and paraffin sections of tissue fixed in various mixtures referred to below. Also, Baker's (1949) method, with formaldehyde-fixed, postchromed, gelatine-embedded sections.

(2) Sakaguchi's (1925) test for arginine, according to the method adapted to histochemical use by Baker (1947a).

(3) Baker's (1946) acid haematein and pyridine extraction test for phospholipids.

(4) The coupled tetrazonium reaction of Danielli (1947, 1950), the technical details being those described by Pearse (1953).

(5) The Feulgen reaction (hydrolysis for 15 minutes at 60° C.). The tissue was fixed in Altmann's or Mann's fluid or in Heidenhain's mercuric-saline.

(6) The periodic acid / Schiff reaction of McManus (1946) and Hotchkiss (1948). The former's $\frac{1}{2}\%$ watery solution of periodic acid was used for 5 minutes. The additional techniques of acetylation and of subsequent saponification in weak alkali, before the reaction, were also employed (McManus and Cason, 1950).

(7) Examination for glycogen. Paraffin sections of tissue fixed in Rossman's fixative were treated by the periodic acid / Schiff technique, some being previously treated with salivary amylase and commercial diastase for 1–2 hours at 37° C. After treatment with the enzymes the slides were covered with celloidin before proceeding further (Lillie and Greco, 1947).

(8) The lead tetra-acetate / Schiff reaction (Hashim and Acra, 1953), combined with the acetylation technique of McManus and Cason (1950).

(9) The Schiff reaction after sodium bismuthate oxidation. Rigby's (1950) solution was employed, viz:

Sodium bismuthate	2 gm.
Glacial acetic acid	10 ml.
Distilled water	10 ml.

Sections of Champy-fixed tissue were brought to water and placed in this

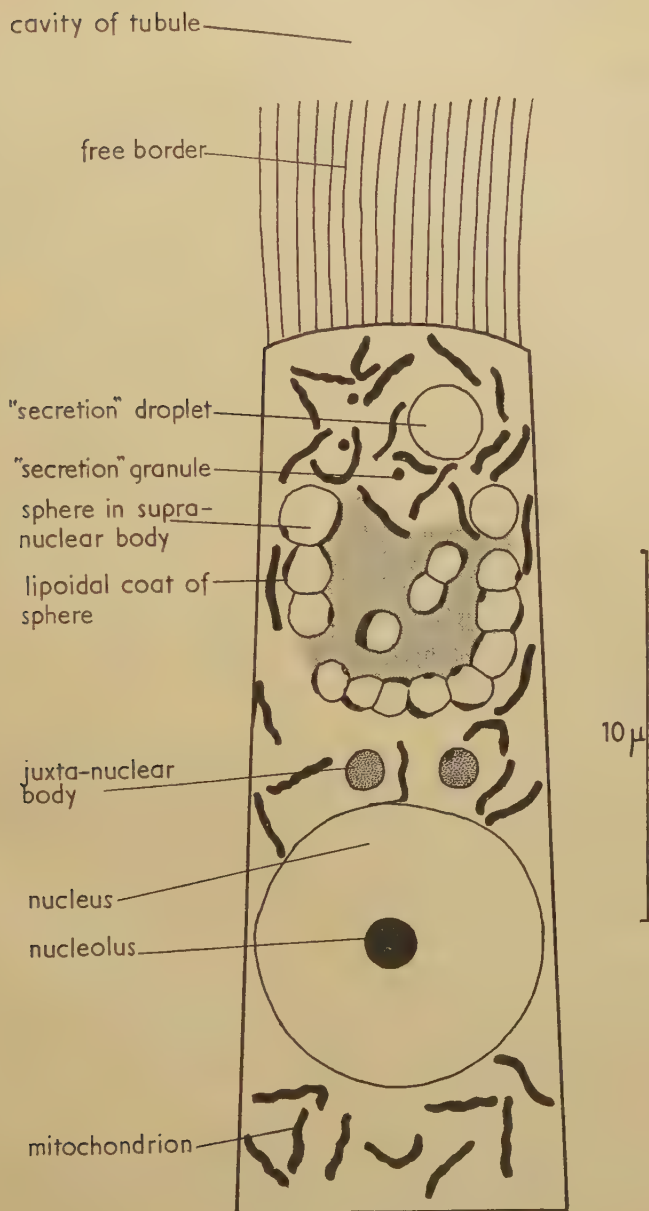


FIG. 1. Diagram of the epithelial cell that lines the storage section of the head of the epididymis of the mouse.

solution for half an hour with constant agitation. After thorough washing they were subsequently treated with Schiff's reagent.

RESULTS

Juxta-nuclear bodies

In the region of the nucleus, and generally above it but occasionally below, spheres or spheroids are observed. They vary somewhat in size. Most of them are about 1μ or less in diameter, but occasionally they almost attain 2μ , especially when situated basally. Sometimes the larger ones are irregular in shape, having a crenated periphery spotted with one or more small deposits of reduced osmium. This appearance is seen in Mann-Kopsch and Kolatchev preparations, and also after fixation in osmium tetroxide alone.

They were observed by Benoit (1921), Nassonov (1924), and Ludford (1925). Benoit considered them to be lipid in nature and all three workers believed that they arise from the nucleolus. Ludford referred to them as 'complex granules', and stated that they occur not only in and above the region of the cytoplasm occupied by the Golgi element but also between the latter and the nucleus. In my material they are mostly adjacent to the nucleus and mainly above it; hence the name I have given them.

They are present in paraffin and gelatine sections of tissue fixed in Altmann's and Champy's fluids, 2% osmium tetroxide, formaldehyde-calcium, cold acetone, and Mann's fluid. They are clearly seen in unstained sections of tissue fixed in the first three fixatives just mentioned, being slightly darkened by the first two and considerably so by the third. Before applying many of the histochemical techniques it was found necessary to treat sections with periodic acid for 5 minutes to bleach them; this completely decolorizes the bodies, no matter how dark previous fixation in osmium-containing fixatives has rendered them. Champy's and Altmann's fixatives give the best morphological detail, and wherever possible sections thus fixed have been employed.

After appropriate fixation they can be coloured even in paraffin sections with Sudan black B or its acetylated derivative. The sudanophilia is alcohol-labile.

With Baker's (1946) acid haematein test, a thin rim of positively-reacting phospholipid is present round the periphery of each body. In life the phospholipid is presumably distributed throughout the bodies, mixed with the other constituent. After pyridine extraction the test is negative.

The bodies are not fixed by hot acetone.

After oxidation by periodic acid (McManus, 1946; Hotchkiss, 1948; Shimizu and Kumamoto, 1952; Glegg and others, 1952; Lhotka, 1952; Hashim and Acra, 1953), or sodium bismuthate (Rigby, 1950), the juxta-nuclear bodies give a strongly positive reaction with Schiff's reagent. Without preliminary oxidation there is no reaction.

The juxta-nuclear bodies are not stained by the coupled tetrazonium reaction. The Sakaguchi test is also negative.

These results, taken together, indicate that the juxta-nuclear bodies consist mostly of cerebroside, with some phospholipid.

The bodies are visible and present a black rim in 'Golgi' preparations made by the Mann-Kopsch and Kolatchev methods, but they are not seen in Aoyama preparations.

Histochemically, the juxta-nuclear bodies contrast strongly with nucleolar material, as the following table shows.

TABLE I

<i>Test</i>	<i>Nucleolar material</i>	<i>Juxta-nuclear bodies</i>
Baker's acid haematein test:		
(a) first part	Moderately strong positive reaction	Positive rim only
(b) second part	Very strong positive	Not present
Feulgen reaction*	Positive (partially)	Negative (completely)
Reduction of 2% osmium tetroxide employed as fixative	Slight	Strong
Sudan black	Not coloured	Strongly coloured
Schiff reaction after oxidation with:		
periodic acid	Negative	Positive
lead tetra-acetate	"	"
sodium bismuthate	"	"
Coupled tetrazonium reaction	Positive	Negative
Mann-Kopsch technique	Visible but colourless	Black
Kolatchev's technique	" "	"

* Performed on paraffin sections of tissue fixed in Altmann's, Zenker's, and Mann's fluids.

Supra-nuclear body

Between the nucleus and the free border of the cell there is a specialized area of the cytoplasm. This area, which is usually roughly spherical, is about 6μ in diameter. Sometimes it is more elongated in the long axis of the cell, up to 10μ . In this area there are numerous spherical or spheroid bodies of various sizes up to about 1.5μ in diameter. These spheres are often arranged in rows, generally slightly wavy. The wavy rows are usually arranged in the long axis of the cell. Sometimes the row is shaped like a horseshoe, with the convex side of the curve pointing towards the nucleus.

None of the staining or histochemical techniques used coloured these spheres, and there is therefore no positive information about their chemical composition in life.

Each sphere is surrounded by a complete or incomplete investment of material that reacts positively to Sudan black. When the investment is incomplete it appears in optical section as a crescent. The black colour is quickly removed by 70% alcohol. The investment therefore contains or consists of lipid. Among all the other histochemical tests used, only the periodic acid / Schiff method gives a positive reaction at or near the surfaces of the spheres.

The whole region in which the spheres lie reacts to dyes and histochemical tests somewhat differently from the ground cytoplasm of the rest of the cell. With basic dyes (toluidine blue and Nile blue) this region stains less strongly than the general cytoplasm; it colours also by the periodic acid / Schiff method, but less intensely than the rims round the spheres. The coupled tetrazonium reaction is positive, resembling that in the general ground cytoplasm. It appears that there is a diffuse substance between the spheres. This substance contains protein and probably carbohydrate (but apparently not glycogen).

When the tissue is fixed in Mann's or Champy's fluid and post-osmicated, much osmium is precipitated in this specialized area of the cell. It is seen in the form of strands, which appear to correspond to the rows of granules. When the sections are treated for 10–15 seconds with $\frac{1}{2}\%$ aqueous periodic acid solution, part of the black material is oxidized and thus removed, and the appearance then approaches more closely to that given by Sudan black, for rims and crescents are seen. It would appear that the osmium is most strongly deposited on or in the lipid rims of the spheres, but also extends between these in a diffuse manner so as to join them. The substance of the spheres themselves does not reduce osmium in the techniques.

The general appearance given by the silver technique of Aoyama is in general similar to that given by the osmium methods, but the fixation is not so good.

An interesting figure is produced if the tissue is fixed in 1% cadmium chloride, embedded in gelatine, and coloured with Sudan black. The lipid rims of the spheres have now run together to form a tubular structure colouring with Sudan black; the centre of the tube is uncoloured.

'Secretion' products

In the upper part of the cell, between the zone just described and the free border of the cell, spheres are frequently seen, larger than those in the supra-nuclear body. These spheres have been regarded by Fuchs (1902) and Ludford (1925) as secretory products. They have generally been regarded as derived from the spheres in the supra-nuclear zone (see Ludford, 1925; Bowen, 1926). For a discussion as to whether they are in fact secretory products or absorbed material, see Wagenseil (1928). None of the stains or histochemical tests used in the present investigation coloured these bodies. They are presumably aqueous, and contain little or no organic matter.

In the same region of the cell as the large spheres just described, small granules are often demonstrable. They are too small for accurate measurement, but may be about $\frac{1}{4}$ to $\frac{1}{2}\mu$ in diameter. They withstand various fixatives (Rossman, Champy, Altmann) and are seen in both gelatine and paraffin sections. The only histochemical tests that showed them were the coupled tetrazonium, periodic acid / Schiff, and lead tetra-acetate / Schiff tests. The coupled tetrazonium test showed them slightly darker than the ground cytoplasm. The Schiff reactions were strongly positive, even after saponification

of acetylated sections. Digestion with saliva or commercial diastase for 1 hour at 37° C. did not prevent the reaction. The granules presumably contain protein and a carbohydrate other than glycogen. These bodies appear to correspond with the 'secretory granules' of Ludford (1925), previously seen by Fuchs (1902). Nicander has recently (1954) described glycogen granules in this region of the corresponding cell of the dog.

Mitochondria

The mitochondria are mostly rod-shaped and slightly wavy. They are well seen in Altmann-Metzner preparations, and also, from their phospholipid content, after acid haematein. Where the mitochondria happen to be closely aggregated together, they are seen to give a positive reaction with the coupled tetrazonium test. They are most numerous in the apical part of the cell, between the supra-nuclear body and the nucleus, and below the nucleus; that is to say, they occur where not excluded by other cytoplasmic inclusions.

DISCUSSION

There is histochemical evidence indicating that the predominant epithelial cell in the head of the epididymis of the mouse contains spheres near the nucleus, consisting mainly of cerebroside but also in part of phospholipid. Bodies of similar nature have previously been reported in the nerve-cells of the thoracic ganglia of the locust (Shafiq and Casselman, 1954) and the sympathetic nerve-cell of the rabbit (Baker and Casselman, 1955). There is no histochemical support for the contention that they are nucleolar extrusion-products. It is possible that spheres ('lipochondria') consisting of cerebroside and phospholipid may be widely distributed cytoplasmic inclusions of diverse cells. It would appear profitable to search for them elsewhere.

Between the nucleus and the free border of the cell there is a supra-nuclear body consisting of a diffuse matrix containing carbohydrate and protein, interspersed with (and partly encompassed by) spheres that are unstained by any staining method or histochemical technique used in the present investigation. These spheres are usually surrounded by rims or capped by crescents of lipid material reacting positively to the periodic acid / Schiff technique, and therefore presumably containing cerebroside. In post-osmication techniques, osmium is deposited on the lipid rims or crescents, and to a less extent on the diffuse matrix.

Between the supra-nuclear body and the free border of the cell, droplets and granules are present, the former containing material that gives no histochemical reactions for organic material, and the latter, much smaller in size, consisting of both protein and carbohydrate. These have been considered to be two types of secretion product by Fuchs (1902) and Ludford (1925), but others, notably Wagenseil (1928), have considered the possibility that the droplets may be absorption products.

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communication, and acknowledges with pleasure much helpful advice and discussion.

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A Cytological Study of the Metabolism of Iron in the Leech, *Glossiphonia complanata*

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With one plate (fig. 1)

SUMMARY

Glossiphonia complanata is found to contain iron in the form of haemosiderin in the gut-content, in the cells of the intestinal epithelium, the coelomic epithelium, the connective tissue, and the epidermis. The haemosiderin is largely in granular deposits.

The amount of iron in the body is reduced on starvation; granular deposits do not now occur, but a positive reaction is still given by the ground cytoplasm of connective tissue and epidermal cells.

Iron is absorbed in limited amounts when the animal is kept in water artificially enriched with this element. It appears to be concentrated in the coelomic epithelial cells and in the connective tissue.

Feeding with *Planorbis corneus* causes significant increase in the iron content of the body of the leech; no such increase is apparent after feeding with *Limnaea stagnalis*.

INTRODUCTION

ALTHOUGH problems of iron metabolism have been studied in great detail in the vertebrates, particularly man, little attention has been paid to the invertebrates. A notable exception is the work of Wigglesworth (1943) on the reduviid bug *Rhodnius prolixus*.

Little appears to have been published on the histochemistry of *Glossiphonia*. Voinov (1928) and Juga (1931) have both published papers on the pigmentation. Since then there has been only the work of Cain (1947a) on the lipids forming the so-called 'Golgi apparatus' of the epithelial cells of the alimentary canal. He has also published incidental observations on the fat-cells (Cain, 1947b).

In this communication it is proposed to deal only with the location and variation of haemosiderin iron in the body of the leech.

MATERIAL AND METHODS

Glossiphonia complanata, a rhynchobdellid leech, is especially suitable for cytological and histochemical study. The cells of the body are very large, so that microscopical observation of intracellular structures is relatively easy. Furthermore the animal is abundant in fresh water and easy to keep alive in the laboratory.

In order to study the normal distribution of haemosiderin in the body, animals which had been feeding before capture were used. In such animals it is usually possible to see the full alimentary canal by transparency, and so to

select appropriate specimens. As the variation of iron content was also of interest, it was decided to look at leeches fed on different molluscs. The animals were starved for 8 weeks before the experiment in order to make sure that any metabolic products of the previous food would be absent from the tissues. *Planorbis corneus* and *Limnaea stagnalis* were chosen as food because the former possesses haemoglobin in its blood, whilst *Limnaea* has haemocyanin. The leeches were kept in large jars, each containing the same quantity of tap-water; this was changed every other day. Animals were killed at fixed intervals after the start of the experiment and fixed in formaldehyde-saline buffered to pH 7.5, as recommended by Lison (1953). Sections were prepared in the usual manner and tested for the presence of iron by Perls's method, modified by Gomori (1952). In this method the section is soaked in an acidified solution of potassium ferrocyanide, and iron is revealed as ferric ferrocyanide (Prussian blue).

RESULTS

In well-fed *Glossiphonia* a positive reaction for haemosiderin iron was obtained in the contents of both crop and intestine. The colour was localized in spheres or irregular masses. Granules containing iron were found in the supra-nuclear region of the intestinal epithelial cells (fig. 1, A). These granules, which were small and spherical, did not occur in every cell. The fibroblasts, which are especially numerous in the region immediately beneath the epidermis, also contained haemosiderin granules. The latter were irregular in shape, and considerably larger than those noticed in the intestinal epithelium. Fat-containing connective tissue cells possessed similar granules, but in addition there was a considerable positive reaction in the ground cytoplasm of the cell (fig. 1, B). In no case did the nucleus give a positive reaction to the test for iron. The cells of the coelomic epithelium (the 'acidic cells' of Kowalevsky (1897)), noticeable because the greater part of their cell-body projects into the coelomic sinus, were found to contain iron only around the periphery of their cell-bodies.

The epidermis showed a positive reaction, which was confined to the cytoplasm of the simple epithelial cells (fig. 1, C); gland cells were entirely negative. No haemosiderin in the form of granules was seen in the epidermal cells. Some of the pigment granules in the large pigment cells gave a weak positive reaction for iron. Often this reaction was so weak that there was doubt whether it could be regarded as truly positive.

FIG. 1 (plate). A, granules containing iron in the intestinal epithelium.

B, fat-containing connective tissue cells, showing haemosiderin granules and positive reaction in the ground cytoplasm.

C, epidermis, showing positive reaction in the cytoplasm. Note that the large gland cells contain no iron.

D, T.S. *Glossiphonia* fed on *Planorbis*. The region of the lateral coelomic sinus, showing many connective tissue cells containing iron.

E, T.S. *Glossiphonia* fed on *Limnaea*. The region of the lateral coelomic sinus, showing absence of iron.

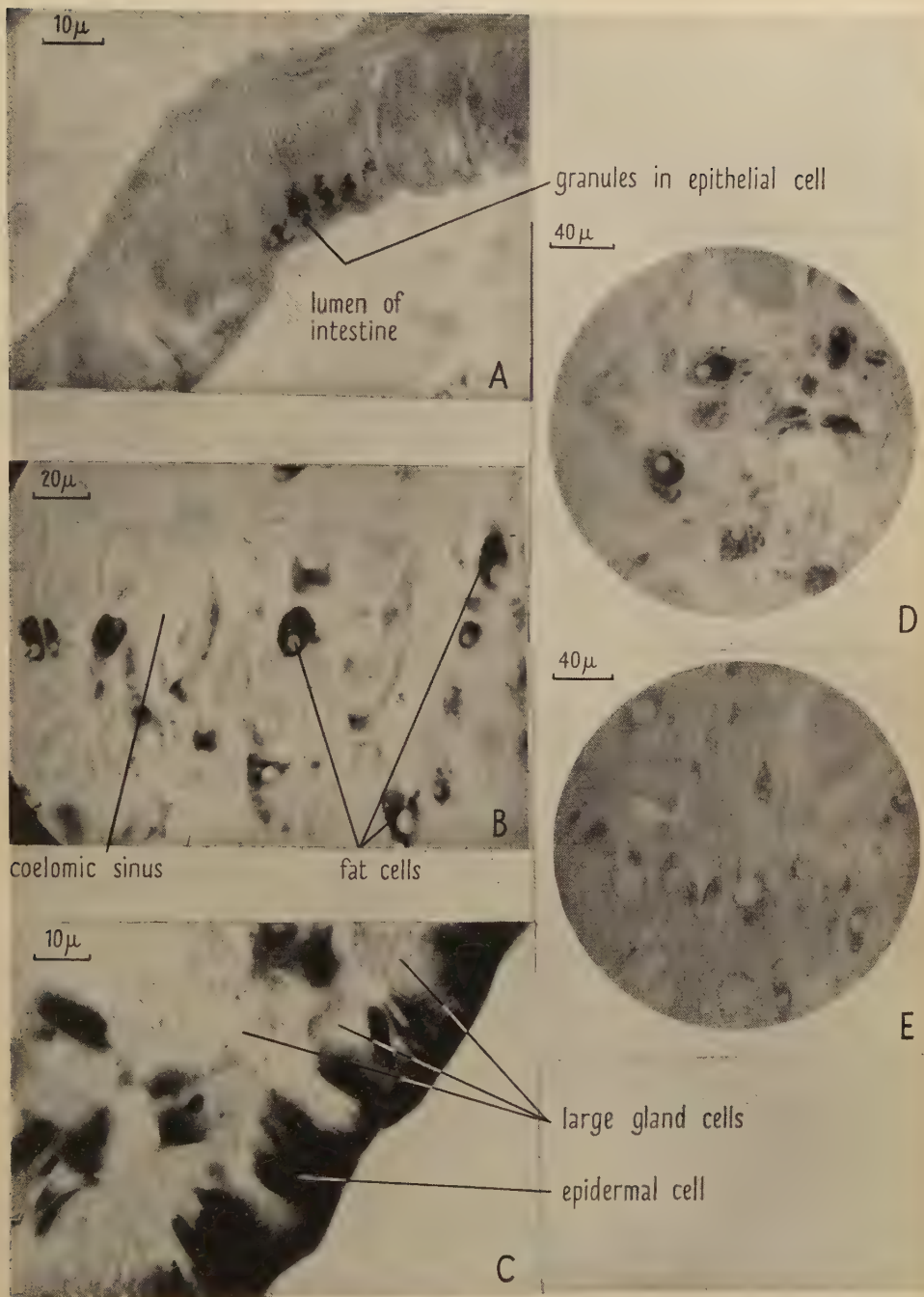


FIG. 1
S. BRADBURY

The location of iron in those animals subjected to various experimental procedures is summed up in table 1.

DISCUSSION

It is to be expected that *Glossiphonia* will take in much haemoglobin with its food, since it feeds largely on *Planorbis*. On digestion, the haemoglobin will be split into globin and haematin, and the latter into proto-porphyrin (which may be transformed into a bile-pigment) and inorganic iron. Owing to the large amount of haemoglobin ingested at any time, the greater part of the inorganic iron thus produced must be presumed to be superfluous to the immediate requirements of the animal. Concentration of iron in the connective tissue cells and epidermis may therefore be a preliminary to its excretion.

TABLE 1

The plus signs indicate the intensity of the reaction to Perls's technique (maximum +++++). The minus sign indicates that no reaction was given.

Tissue or cell	Location of haemosiderin	Control	Fe+++ added to water	Fed on Planorbis	Fed on Limnaea
Epidermis . . .	Ground cytoplasm	Weak +	+	+	Weak +
Fibrocytes . . .	Granules	Few +	++++	++++	—
Fat cells . . .	Ground cytoplasm	+	++	++	—
" " . . .	Granules	—	—	++++	—
Hyaline connective tissue	Matrix	—	+	—	—
Coelomic epithelial cells .	Ground cytoplasm	—	+	Weak + Some —	—
Coelomic leucocytes .	Granules	—	+	—	—
Large pigment cells .	Pigment granules	+	+	+	+

Kowalevsky (1897) and Juga (1931) have both shown that the coelomic leucocytes and coelomic epithelial cells are capable of absorbing particles of carmine injected into the coelomic fluid. These same cells can be shown to contain iron when the animal is kept in water of high iron content. This observation seems to indicate that this metal is present in the coelomic fluid; it may possibly pass in solution through the epidermis into the hypodermal coelomic canals, and thus into the general coelom.

It is not possible at this stage to say whether the granules noticed in the intestinal epithelial cells represent iron in the process of absorption. It may well be that these cells are acting as a kidney of accumulation. An analogous case is found in *Rhodnius*, where Wigglesworth (1943) has shown that similar cells are acting as a storage centre for inorganic iron.

Starvation for a period of 8 weeks has the effect of reducing appreciably but not abolishing the positive reaction of the tissues to Perls's test for iron. One striking feature of the starved leech is that the large granular deposits of iron

are absent. It seems likely that some fraction of the iron present is bound to the tissues, possibly by linkage to some form of protein substrate.

It is apparent that some iron is absorbed when *Glossiphonia* is kept in water with a high iron content. The element appears to be concentrated in the connective tissue and coelomic epithelial cells, and also to some extent in the epidermis. Although the hyaline matrix surrounding the connective tissue cells shows a weak positive reaction to the test for iron, undue emphasis must not be placed on this observation, as it may be a diffusion artifact.

The results of feeding with different molluscs are well marked (fig. 1, D and E). When *Glossiphonia* is fed on *Limnaea*, no increase in the iron content of the body is observed. With *Planorbis* as food, on the contrary, there is a great increase in the body iron content, particularly in the connective tissue cells, where it appears to be deposited in a granular form. There does not appear to be much increase in the iron content of the coelomic epithelial cells, or of the epidermis. This forms an interesting contrast with the leech kept in water to which a ferric salt has been added: these particular tissues then show an increase in iron content. The difference could be explained by postulating that in the case of a leech fed on a diet rich in iron, the process of absorption of this element proceeds in such a way that it never enters the coelomic fluid.

It seems clear from these results that some correlation exists between the iron content of the tissues of the leech and the nature of its diet.

I wish to express my appreciation of the help I have received from Prof. A. C. Hardy, who allowed this work to be carried out in his department. In addition, I am indebted to Dr. J. R. Baker, to Dr. A. J. Cain, and to Dr. S. M. Russell for invaluable advice and discussion. The photomicrographs were made by Dr. Baker and Miss B. M. Jordan.

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Haemoglobin in the Fat-Cells of *Daphnia*

By J. GREEN

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SUMMARY

When placed in well aerated water, *Daphnia* can lose haemoglobin from its blood by two methods. The haemoglobin may be passed into the eggs or it may be broken down in the fat-cells. When populations of *D. curvirostris* or *D. pulex*, with much haemoglobin in the blood, are placed in conditions in which they can produce very few eggs and are then placed in well aerated water, haemoglobin appears as globules in the fat-cells. This appears to be an emergency measure to remove haemoglobin from the blood until the fat-cells can break it down. When equally red animals, producing ten eggs each, are placed in well aerated water, globules of haemoglobin do not appear in the fat-cells. Sufficient haemoglobin is drained from the blood into the eggs to render the formation of haemoglobin globules in the fat-cells unnecessary.

LANKESTER (1871) discovered that the red colour of the blood of certain species of *Daphnia* is due to haemoglobin. The amount of haemoglobin in the blood differs in different species and in the same species under different conditions. The blood of an individual may change from colourless to bright red and may then become colourless again. These changes are brought about mainly by changes in the oxygen content of the water in which the animal is living (Fox, 1948). When the oxygen content of the water is low there is much haemoglobin in the blood of *Daphnia*; when oxygen is plentiful very little haemoglobin can be detected.

Haemoglobin is also present in the parthenogenetic eggs of *Daphnia* (Teissier, 1932), and may be found in the muscles, brain, and fat-cells (Fox, 1955). These last cells have been described in some detail by Jäger (1935), particularly in relation to the storage of fat. More recently Smaridge (1954) has shown that when *Daphnia* is losing haemoglobin, iron appears in these cells. During the many years that various species of *Daphnia* have been used for experiments in this laboratory haemoglobin has only been found in the fat-cells on a few occasions, and then only in small amounts. It was a considerable surprise therefore when a number of females of *D. curvirostris* Eylmann were found with bright red globules of haemoglobin in their fat-cells. When examined with a microspectroscope, absorption bands of oxyhaemoglobin were much stronger in these cells than elsewhere in the body. These animals had been kept in a dish together with a sample of *D. magna* Straus and a few specimens of *D. pulex* (de Geer). The Cladocera had at first been very numerous, and the crowded conditions had reduced egg-production so that very few were carrying eggs. As conditions in the dish deteriorated a great many of the Cladocera died, leaving a much smaller number in less crowded conditions. In these less crowded conditions the oxygen content of the water

would be higher and so haemoglobin loss would ensue. Now *Daphnia* can lose a considerable amount of haemoglobin from its blood by passing it into its eggs (Dresel, 1948). The work of Smaridge (1954) indicates that haemoglobin breakdown occurs in the fat-cells. Since haemoglobin is removed from the blood when the oxygen content of the surrounding water increases, when *Daphnia* is producing few or no eggs a greater load will be placed on the fat-cells. The appearance of globules in the fat-cells seems to be an emergency measure that operates until the cells can break down the haemoglobin.

Although all the specimens of *D. curvirostris* and *D. pulex* which were examined had haemoglobin in their fat-cells, not one specimen of *D. magna* was found in this condition. All three species had been taken from the same locality and kept in the same dish. The initial haemoglobin index (cf. Fox, 1948) of *D. curvirostris* when brought into the laboratory was 96; that of *D. magna* was 44. When the globules were first noticed the haemoglobin index of *D. curvirostris* had fallen to 40. The higher initial index of *D. curvirostris* adds weight to the idea that the appearance of haemoglobin globules is connected with an increased load on the fat-cells during haemoglobin breakdown.

After making these preliminary observations an attempt was made to confirm the ideas outlined above. Two populations of *D. pulex* from the district around Hillerød, Denmark, were used. Both were bright red with haemoglobin in the blood, but one was producing very few eggs whereas in the other the females were carrying an average of ten eggs each. Groups of ten females from these populations were placed in open dishes containing 120 ml. of water; a few millilitres of pond water were added to each dish to provide a small amount of food. The dishes were kept at room temperature which varied between 16° and 20° C. The oxygen content of the water in these dishes was not measured, but measurements made on similar dishes with the same number of animals show an oxygen content of between 5 and 6.5 ml. per litre.

The population which was producing an average of ten eggs per female lost a large amount of haemoglobin without any appearing as globules in the fat-cells. The haemoglobin index fell from 91 to 50 in 5 days.

In the population with few eggs small globules of haemoglobin appeared in the fat-cells after 2 days in the open dishes (fig. 1, A). After 3–4 days the size and number of the globules increased; the cells also increased in size (fig. 1, B). After 4 days some of the large globules began to show irregularities and to look like partially deflated balloons (C); colourless globules appeared in some of the cells at this time (D). In some individuals these colourless globules appeared after only 3 days. When 7 or 8 days had passed most of the fat-cells had lost nearly all their haemoglobin; only a few small globules remained. The cells did not become smaller, but remained large. In these later stages the nuclei of the fat-cells became clearly visible (E). There was a great variation in the rate at which different individuals completed the

process; some went through the whole cycle in 5 days, in others a few globules of haemoglobin were still present after 12 days.

Throughout the process the haemoglobin was in separate vacuoles, never

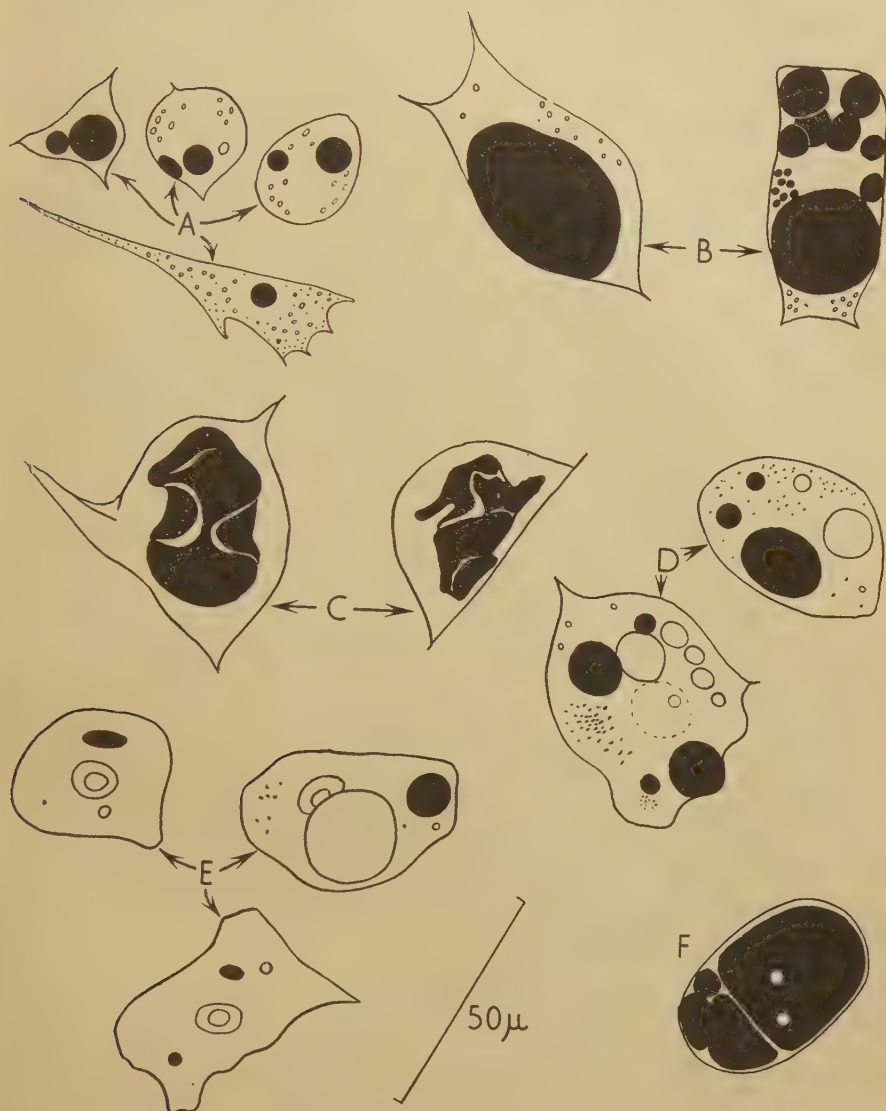


FIG. 1. Haemoglobin in the fat-cells of *D. pulex* after transfer to shallow dishes. A, after 2 days. B, after 3 days. C, after 4 days. D, after 4 days, showing clear globules. E, after 8 days. F, fat-cell from late-stage embryo. The haemoglobin is black in the figures, which are all drawn to the same scale.

in the cytoplasm of the cells. No coloured products were found during the disappearance of the haemoglobin, thus lending support to the suggestion

made by Smaridge (1954) that the haem is broken down beyond the porphyrin or bile pigment stage to simpler colourless compounds. The haemoglobin index of this population with few eggs fell from 96 to 48 in 5 days. This is practically the same rate of loss from the blood as in the population producing an average of ten eggs per female. This latter population had lost its haemoglobin from the blood without any appearing in the fat-cells. The production of ten eggs appears to drain enough haemoglobin from the blood to render the formation of haemoglobin globules in the fat-cells unnecessary.

Further support for the idea that the appearance of haemoglobin in the fat-cells is due to an increased load being placed on these cells came from observations made on the embryos produced by these two populations of *D. pulex*. When ten embryos per female were present the haemoglobin was located in the embryonic blood, such as is normally the case. When only one egg was present in the brood pouch of each female, much more haemoglobin was passed into this egg than into each of the ten eggs. The single eggs were markedly redder. As these single embryos developed, some of the haemoglobin remained in their blood, but a large amount passed into globules in the fat-cells (fig. 1, F). These globules were still present when the young were liberated, but disappeared after 3 or 4 days.

It thus seems that three conditions are necessary for globules of haemoglobin to appear in the fat-cells of *Daphnia*. The animals must have a large amount of haemoglobin in the blood, they must be producing very few or no eggs, and must then be transferred to well aerated water. The phenomenon described in this paper is a response to a stimulus to remove haemoglobin from the blood at a rate greater than that at which the fat-cells can destroy haemoglobin.

The original observations on *D. curvirostris* were made at Bedford College, London, in the department of Prof. H. Munro Fox, who checked the spectroscopic examination of the cells and has kindly read and criticized the manuscript. The observations on *D. pulex* were made at the Freshwater Biological Laboratory of the University of Copenhagen, where Prof. Kaj Berg was most hospitable. The work in Denmark was aided by a grant from the Central Research Fund of London University.

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A Simple Pyronine / Methyl Green Technique

By BARBARA M. JORDAN AND JOHN R. BAKER

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SUMMARY

Brachet's pyronine / methyl green technique, which gives good results when made with certain foreign dyes, does not work well when British specimens of pyronine and methyl green are used. Instructions are given for making up and using a reliable staining solution containing dyes that are readily available in this country.

FOR his pyronine / methyl green (P/MG) technique, Brachet (1953) recommends the pyronine of Geigy and of Grübler and the methyl green of Anachemia, Grübler, and Ciba. In trying his method we used dyes sold by English firms. Our results were not satisfactory. The pyronine was largely extracted during the 5 minutes' stay in 95 per cent. alcohol, and the methyl green did not stain well except in the internal part of the piece of tissue.

We had been engaged in an attempt to improve the P/MG technique when Brachet's paper was published, and our failure to get good results with English dyes when we tried his technique caused us to revert to this subject. In this work we have used the dyes of Messrs. G. T. Gurr (pyronine G 6104, methyl green 05563).

Our work has been carried out on the following tissues: skin, stomach, intestine, submaxillary gland, liver, pancreas, kidney, and anterior mesenteric ganglion of mammals (mouse, cavy, and rabbit); pancreas, leg-muscle, and cerebellum of the frog; cerebral ganglion of *Helix pomatia*; ovary and ventral ganglia of the earthworm; and transverse sections of the leech, *Glossiphonia complanata*. We have found the pancreas of the mouse the most useful test-object for comparing the effects of variations in technique.

The staining solution that has given us the best result is made thus:

Extract 0.5 per cent. aqueous methyl green solution repeatedly with chloroform until the chloroform is nearly colourless. At least eight extractions are necessary.

Make an acetate buffer at pH 4.8 by mixing 81 c.c. of N/5 acetic acid with 119 c.c. of M/5 sodium acetate.

Prepare the stain as follows:

Pyronine, 0.5 per cent. aq.	37 c.c.
Methyl green, 0.5 per cent. aq. (extracted)	13 c.c.
Acetate buffer at pH 4.8	50 c.c.

This solution, which can be used at once, still stains well four months after preparation.

The pyronine in our solution is considerably weaker than in Unna's (1913)

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and Brachet's solutions, and slightly weaker than in Trevan and Sharrock's (1951). The methyl green in our solution is much weaker than in Unna's and Brachet's (less than half the concentration), though somewhat stronger than in Trevan and Sharrock's.

Our technique is as follows:

- I. Fix a piece of tissue not exceeding 3 mm. in thickness in Zenker's fluid for 3 hours.
- II. Wash in running water for 24 hours.
- III. Embed quickly in paraffin through alcohol and toluene.
- IV. Cut sections at 7μ .
- V. Bring sections to water in the ordinary way (passing through iodine and sodium thiosulphate solutions on the way, in accordance with the usual practice after Zenker fixation). Wash for a few minutes in running water.
- VI. Rinse in distilled water.
- VII. Blot with filter-paper.
- VIII. Stain for $\frac{1}{2}$ hour in the buffered P/MG.
- IX. Rinse for a few seconds in distilled water.
- X. Quickly blot nearly dry.
- XI. Acetone (to dehydrate), 1 minute.
- XII. Pass through 1:1 acetone-xylene into xylene, and mount in D.P.X. (or other neutral mounting-medium).

Result: chromatin, blue, blue-green, or green; nucleoli and basiphil cytoplasm, red. The material stained red can only be interpreted with certainty as ribonucleic acid if tests are made with ribonuclease.

We tried a large number of fixatives, and found that 3 hours in Zenker's fluid gave the best fixation that was compatible with characteristic staining by P/MG.

The method presents no technical difficulties.

Kurnick (1952) found that dehydration in acetone removed pyronine after P/MG staining, and that it was necessary to re-stain in a saturated solution of pyronine in acetone. He used pyronine B. We do not find any restaining to be necessary with the specimen of pyronine G that we have used. It seems doubtful whether pyronine gives characteristic staining results unless it is ionized in aqueous solution.

It is worth remarking that our P/MG solution can conveniently be used for showing the macronucleus of *Paramecium*. Put a drop of water containing the living ciliates on a slide. Add a drop of P/MG. Mix the fluid by drawing it gently into a pipette and pressing out again several times. Leave for 15 minutes. Cover. Result: macronucleus, green; cytoplasm, pink. The method is very suitable for use in elementary classes in zoology.

Rhodamine S can be substituted for pyronine in this technique, as one of us showed many years ago (Baker, 1942). This dye is more powerful than pyronine, and the concentration of the stock solution should be reduced from

0.5 to 0.25 per cent. We have used the product of the British Drug Houses Ltd.

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Further Studies of the Third Instar Larval Cuticle of *Calliphora erythrocephala*

By L. S. WOLFE

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Department of Agriculture, London, Ontario)

With one plate (fig. 1)

SUMMARY

The penetration and reduction of ammoniacal silver nitrate solution in the epicuticle of the larva of *Calliphora* was studied. The epicuticle of the third instar larva is more permeable over the muscle insertions and cuticular sense organs. This finding is related to their development at the previous moult.

A surface layer of orientated wax is not present. Proteinaceous and fatty materials from the feeding medium modify the properties of the cuticle surface. Chloroform-methanol extracts a soft light brown acidic lipide from the protein of the epicuticle after contaminants from the medium are removed.

The water loss from larvae and puparia of different ages and after various treatments was studied. Young puparia recover from abrasion but larvae do not. An hypothesis that waxy substances are liberated on to the surface of the puparium during hardening and darkening of the cuticle is presented and discussed.

The pore canals penetrate the endocuticle until they are cut off from the epidermis by the development of the prepupal cuticle just after the puparial contraction. An inner endocuticle in which pore canals were absent was not found. The structure of the pore canals as shown by phase contrast examination is discussed. The pore canals are three times more concentrated in the lateral regions than in the dorsal or ventral regions.

The oenocytes go through a secretory cycle during puparium formation similar to that occurring before moulting of the larva.

INTRODUCTION

IN the course of a study of the deposition of the third instar larval cuticle of *Calliphora erythrocephala* Meigen (Wolfe, 1954) some new observations were made on the structure and properties of the cuticle. This paper reports these findings.

MATERIALS AND METHODS

The rearing of the larvae and the histological methods were the same as described previously (Wolfe, 1954). Ammoniacal silver nitrate solutions were freshly prepared before use. Water loss through the cuticle was studied using the methods of Wigglesworth (1945). The phase contrast microscope was used for studying the structure of the pore canals and the oenocytes. Special techniques are described at the appropriate places in the text.

The occurrence and distribution of reducing substances in the cuticle

Wigglesworth (1945, 1948) used ammoniacal silver nitrate solution to demonstrate the presence of reducing polyphenols in the insect cuticle and

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also to show the extent of damage to the epicuticle after abrasion. This method was used in this study not only to demonstrate the presence of reducing substances in the larval epicuticle of *Calliphora*, but also as a qualitative indication of regions in the cuticle more readily penetrated by aqueous solutions.

Third instar larvae of different age groups were immersed in a 5 per cent. ammoniacal silver nitrate solution at room temperature for 6 hours and thoroughly washed with distilled water. When examined under a binocular microscope, a series of well-marked deposits of silver were observed in the outer layers of the cuticle. These deposits were localized in feeding larvae in regions of the muscle insertions, cuticular sense organs, and functional and vestigial spiracles. In mature larvae irregularly distributed deposits were found particularly in the spinous regions and were not associated with sense organs or muscle insertions. Sections of the cuticle through these regions revealed that they were produced by small lesions in the outer epicuticle. These lesions were probably produced by the spines tearing and scratching the outer epicuticle during the muscular contortions of the larvae while feeding. Though probably present in young larvae they were not revealed by reduction of silver solutions. Dennell (1947) showed that reducing substances appeared in the inner epicuticle only in mature larvae.

Short periods of immersion in ammoniacal silver solution (2-3 hours) resulted in clusters of small deposits of silver at the muscle attachments (fig. 1, A). These deposits were restricted to the outer epicuticle. After longer immersion periods (12 hours) the solution penetrated through the endocuticle and was reduced at the base of the tonofibrillae and the surrounding epi-

FIG. 1 (plate). A, reduction of ammoniacal silver nitrate solution by the tips of the tonofibrillae of the muscle attachments in the epicuticle of a mature third instar larva. Immersion time, 2 hours.

B, oblique section through a cuticular sense organ of a mature larva after immersion in ammoniacal silver solution for 4 hours.

C, surface view of reduction at the cuticular sense organ to show the intensity and extent of the reduction around the sensory peg.

D and E, the intense reduction of ammoniacal silver nitrate solution after abrasion of the outer epicuticle of a larva 12 hours before puparium formation. D, abrasion with fine needle; E, abrasion with powdered glass.

F, sagittal section through the cuticle of the 'white' puparium to show the pore canals extending into the endocuticle from the epidermis and the absence of an inner endocuticle. The oenocytes are shown immediately beneath the epidermis. Osmic acid / Orcein stained; frozen section.

G, the pore canals in the cuticle of a mature third instar larva, 'crop full' stage. The basal and distal portions of the pore canal are easily distinguished. Phase contrast, oil immersion, frozen section.

H, reduction of ammoniacal silver nitrate solution by the tips of the pore canals in the inner epicuticle of a mature larva. Immersion time, 24 hours.

I-N, phase contrast microphotographs of oenocytes at different stages of their secretory cycle before puparium formation. Mounted in *Drosophila* ringer. I, 'crop full' stage; J, 30 hours before puparium formation; K, 20 hours before puparium formation; L, quiescent period just before puparial contraction; M, 'white' puparium stage; N, 5-hour puparium just before the separation of the oenocytes from the epidermal cells.

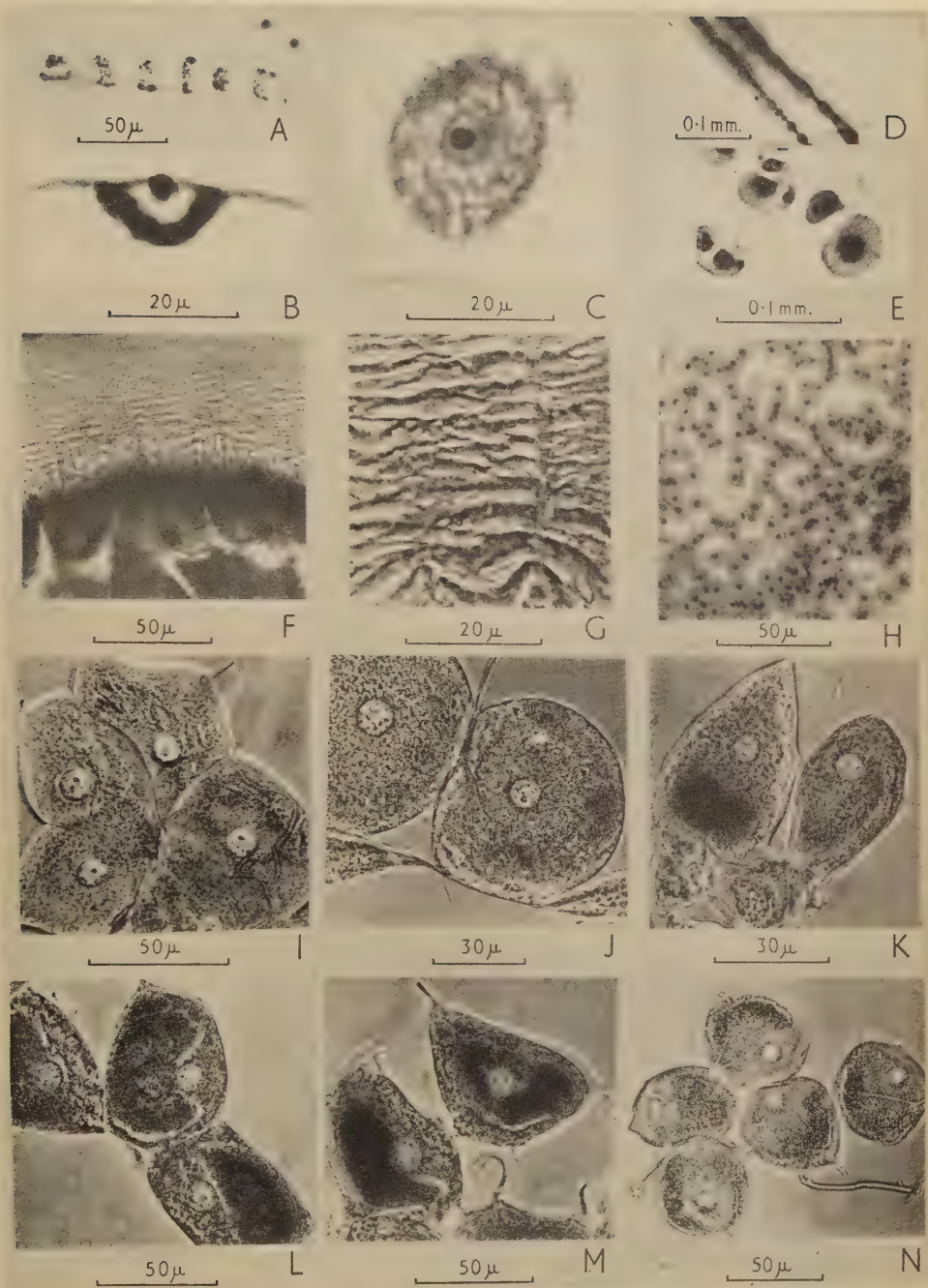


FIG. 1
L. S. WOLFE

dermal cells. No reduction occurred in the endocuticle. In feeding larvae reduction was localized to the sensory pegs of the sense organs, but in the mature larvae the cuticular depression also showed deposits of silver (fig. 1, B and C). Sections of the cuticle through the sense organs after 12-hour immersion periods showed that the silver solution had penetrated through the epicuticle of the sensory pegs and down the distal processes to the sense cells within the epidermis.

The localized reduction of ammoniacal silver solutions by the sense organs and muscle insertions is interpreted as indicating a greater permeability of the epicuticle over these regions. Further confirmation for this conclusion was obtained by immersing larvae for 30 minutes in a saturated solution of cobalt chloride, rinsing in distilled water, and placing them in hydrogen sulphide water. Larvae thus treated showed black patches of cobalt sulphide over the muscle insertions, cuticular sense organs, and spiracles. Addition of detergents (Triton X, Co 9993, Teepol) to the cobalt solution did not increase the size of the sulphide deposits except for a greater depth of penetration down the spiracles.

The modification of the epicuticle over the muscle insertions and cuticular sense organs arises during development at the previous moulting cycle (Wolfe, 1954). The tonofibrillae and distal nerve fibres are attached across the exuvial space to the old cuticle until just before ecdysis and the old epicuticle at this time is penetrated by fine fibres of the tonofibrillae at the muscle attachments and the distal nerve fibres at the sense organs. When the cuticle is shed a break occurs at the region where the tonofibrillae of the old cuticle penetrate the newly formed epicuticle. Similarly, a break occurs at a level just below the sense rods where the distal sensory nerve fibre penetrates the epicuticle of the new sensory peg. It is the tips of the cuticularized fibres of the tonofibrillae and the sensory nerve fibres that are thought to give the quick surface reduction of ammoniacal silver solution.

Kühnelt (1949) reported the presence of reducing spots within the cuticle of insects from widely different groups. He also found deposits at the muscle insertions, dermal sense organs, cuticle lesions, and cuticular pores. No openings of ducts or pores were found within the larval cuticle of *Calliphora* except around the spiracles.

The reducing ability of the muscle attachments and cuticular sense organs is not attributed to phenolic substances. The reduction at the cuticle lesions, however, is very probably due to exposure of polyphenolic substances in the inner epicuticle. Feeding larvae whose cuticles had been abraded either by rubbing in finely powdered glass or scratched with a fine needle showed only slight browning at the abraded areas after immersion in ammoniacal silver solution. However, larvae similarly treated at the 'crop full' stage showed an intense reduction (fig. 1, B and E). Powerful reducing substances are added to the inner epicuticle at this stage. Pryor (1940) and Dennell (1947) have conclusively shown that this strong reduction is due to polyphenolic compounds, probably an *o*-dihydroxyphenol. Mature larvae rubbed in alumina

dust showed no increase in reduction within the cuticle when immersed in ammoniacal silver solution. The outer epicuticle is very resistant and must be deeply abraded to expose the inner epicuticle.

The epicuticle

The staining reactions of the epicuticle of *Calliphora* larvae and the changes in these reactions at puparium formation differed little from the closely related species *Sarcophaga falcata* (Dennell, 1946), and *Rhagoletis cerasis* (Wiesmann, 1938). However, the outer epicuticle stained red in Mallory's stain and black in Heidenhain's haematoxylin. In young larvae the inner epicuticle stained pink in Mallory's stain but in mature larvae became a deep blue and also gave stronger Millon's and ninhydrin reactions. The appearance of phenolic substances and oxidase in the inner epicuticle was found by Malek (1952) to coincide with the presence of more protein.

The protein-lipide association in the inner epicuticle before puparium formation contains all the requisite materials for the formation of the sclerotin of the exocuticle (Pryor, 1940, 1947). Sclerotin formation commences in the inner epicuticle at puparium formation and spreads to the outer endocuticular layers. The term 'exocuticle' should be applied only to sclerotinized cuticle whether it is of epicuticular or endocuticular origin or both. The exocuticle of the puparium consequently includes both endocuticle and epicuticle which become indistinguishable. The outer epicuticle remains distinct, but its pale amber colour suggests that it also contains sclerotin.

The surface of the larval epicuticle is hydrophil. An orientated superficial wax layer on the epicuticle of the type described by Beament (1945) and Wigglesworth (1945) is absent from the *Calliphora* larva. However, lipides are incorporated in the epicuticle. The epicuticle breaks down into oily droplets when treated with concentrated nitric acid and potassium chlorate (cuticulin reaction). Chloroform extracts small quantities of a soft, almost liquid, waxy material of indefinite melting-point from the epicuticle. Beament (1945) calculated a wax thickness of 0.27μ on the washed puparium and 1.1μ on the unwashed larval cuticle. He attributed this difference of wax thickness to the presence of contaminants from the larval environment on the unwashed cuticle. Experiments were performed to investigate the nature of the hydrophil cuticle and the extent the larval environment affects cuticle wettability.

Two hundred unwashed mature *Calliphora* larvae were rolled in alumina dust for 15 minutes, removed from the dust, and washed quickly with a jet of alcohol. The alumina dust was then extracted with 2:1 chloroform-methanol, a solvent mixture that extracts little non-lipide material. This procedure yielded 17.5 mg. of a strongly smelling acidic grease with indefinite melting-point. The yield, after repeating the above procedure with larvae previously washed in distilled water, was only 0.7 mg. An approximate calculation gives a wax thickness of 1μ on the unwashed and 0.004μ on the washed larval cuticle. The latter value is so small that the presence of a superficial wax layer on the epicuticle appears unlikely. The waxy materials

extracted from the unwashed larvae originate as suggested by Beament (1945) from substances in the feeding medium adsorbed on to the epicuticle.

Three separate batches of fifty washed, isolated, and dried cuticles of mature *Calliphora* larvae were weighed and extracted for 1 hour with chloroform-methanol. After removal of solvent, 3.2 ± 0.3 mg. of a soft light brown wax of indefinite melting-point remained representing 1.1 per cent. of the dry cuticle weight. This wax is the true epicuticular lipide of the cuticle.

A mature *Tenebrio* larva floats when dropped into a Petri dish containing distilled water and the epicuticle surface remains perfectly dry. If an unwashed *Calliphora* larva was added to a dish containing a floating *Tenebrio* larva it immediately sank and within 2 minutes the *Tenebrio* larva also sank with its epicuticle completely wetted. Three washed and blotted *Calliphora* larvae placed in a fresh dish containing a floating *Tenebrio* larva sank, but in this case the *Tenebrio* larva remained floating for 30–45 minutes. A *Tenebrio* larva previously placed among unwashed *Calliphora* larvae sank immediately when dropped into a fresh dish of distilled water. These experiments indicate that the contaminants on the unwashed *Calliphora* cuticle can affect the wettability of the *Tenebrio* cuticle. This may result from the formation of a hydrophil film on the cuticle surface, the alteration of the surface tension of the water, or a genuine disruption of the wax layer. Hurst (1941, 1948) observed that *Calliphora* larvae in contact with *Tenebrio* larvae caused the latter to die from desiccation. He stated that the hydrophil free lipides of the *Calliphora* epicuticle disrupted the hydrophobe wax layer of the *Tenebrio* epicuticle. Richards, Clausen, and Smith (1953) discredited this interpretation and concluded that the *Tenebrio* larvae used by Hurst were damaged. The hydrophil free lipides of Hurst are nothing but contaminants from the larval environment and these can alter the surface properties of the *Tenebrio* epicuticle.

Water loss through the cuticle

The transpirational loss of water through the cuticle of larvae and puparia of different ages and after various treatments with their spiracles sealed with Celamel was determined by the methods of Wigglesworth (1945). The results are summarized in table 1.

Water loss from the larva decreased with age (table 1, A). There is a parallelism between this observation and observations of the penetration of aqueous poisons through the cuticle. Second instar larvae were immobilized within 30 seconds when immersed in 0.1 per cent. KCN or HgCl_2 . Mature third instar larvae were immobilized in 20–40 minutes. Ammoniacal silver nitrate solution immediately penetrated the first and second instar cuticles and was reduced at the epidermis. A greater permeability of the early instars to arsenite was shown by Lennox (1940) for *Lucilia cuprina* and by Ricks and Hoskins (1948) for *Sarcophaga securifera*. Speyer (1925) found a 200 per cent. increase in the resistance of *Lucilia sericata* to penetration by poisons after the first 24 hours of the larval period. The increased resistance to water loss and to penetration of aqueous poisons by third instar larvae may be correlated

with epicuticle thickness. In the first and second instars the epicuticle thickness is less than 1μ whereas in the third instar it varies from 3 to 7μ in

TABLE I

Percentage loss of weight of *Calliphora erythrocephala* larvae and puparia after various treatments and exposure to dry air over P_2O_5 for 4 hours at $25^\circ C$.

Object of treatment		Per cent. loss of weight
A.	Second instar larva untreated	6.4
	Third instar larva feeding "	2.9
	Mature larva "	1.8
	White puparium "	1.5
	Puparium 4 hours "	1.6
	Puparium 40 hours "	1.1
B.	Larva 'crop full', control	2.1
	" immersed cold $CHCl_3$ 3 minutes	21.0
	" immersed hot $CHCl_3$ 3 minutes	44.0
	" rubbed with alumina dust; dust left on	3.2
	" heated first to $60^\circ C$.	9.5
	" smeared with Co 9993	2.8
C.	Puparium 2 hours, control	1.3
	" immersed in $CHCl_3$ for 3 minutes	52.4
	" immersed in benzol for 3 minutes	43.1
	" immersed in alc.-ether (3:1)	23.5
	" immersed in acetone for 3 minutes	22.9
	" immersed in abs. alc. for 3 minutes	5.1
D.	Puparium 2 hours, control	1.4
	" surface scraped to damage epicuticle	54.3
	" surface scraped; left 12 hours	3.8
	" surface scraped; left 12 hours; smeared Co 9993	19.4
	" surface smeared Co 9993	22.4
	" rubbed with alumina dust	12.8
	" rubbed with alumina dust; rinsed dist. water; left 12 hours	2.6
E.	Puparium 10 hours, control	1.2
	" surface scraped	46.0
	" puparium removed in section to level of pre- pupal cuticle	30.4
F.	Puparium 35 hours, control	1.0
	" puparium removed post half	2.7
	" puparium removed post half; pupal cuticle smeared with Co 9993	10.5
	" ditto; pupal cuticle immersed in $CHCl_3$ for 3 minutes	21.0
	" immersed with puparium intact in $CHCl_3$ for 3 minutes	1.2

thickness. Also an inner epicuticle cannot be seen in the epicuticle of first and second instar larvae. It may be that the presence of an inner epicuticle is essential for the control of water loss as well as water penetration. Richards, Clausen, and Smith (1953) have recently shown that the inner epicuticle of *Sarcophaga bullata* is essential for the phenomenon of asymmetrical penetra-

tion to occur. The suggestion by Bonnemaison and Cayrol (1951) that the endocuticle thickness is a factor in resistance to penetration of insecticides seems less likely.

Treatment of the larva and early puparium with organic solvents greatly increased the water loss through the cuticle (table 1, B and C). This is almost certainly a result of extraction of waxy substances and disorganization of the epicuticular protein-lipide complex. Larvae rubbed in alumina dust or smeared with the powerful detergent Co 9993 (cetyl ether of polyethylene glycol) showed no increase in water loss. This is further evidence for the absence of an orientated surface wax layer controlling water loss on the larval cuticle of *Calliphora*.

A curious difference was found between the mature larva and the early puparium. Rubbing the early puparium with alumina dust led to a significant water loss, but if the puparium was left for 12 hours impermeability was completely restored (table 1, D). Recovery also occurred after light scraping of the epicuticle of the puparium. The reasons for this recovery reaction are not clear. A possible explanation is that wax is continuously secreted during the darkening and tanning of the puparium. The puparium progressively darkens and hardens during the first 20–25 hours, and this is precisely the period before pupation when recovery from abrasion was observed. However, wax could not have been secreted continuously by the epidermis or from gland cells during this period because they are separated from the puparial cuticle 2 hours after the puparial contraction by the formation of a very thin prepupal cuticle.

Recovery of the larval cuticle from abrasion was not observed. The proteinaceous and waxy materials on the surface of the larval epicuticle derived from the feeding medium are also present as a solidified and oxidized layer on the surface of the puparium. The puparium is not wetted as readily as the larva and also shows a higher resistance to water loss. Rubbing the puparium in alumina dust led to a much greater increase in water loss than in the larva (table 1, D). This indicates that the surface waxy materials on the puparium do control water loss and suggests that besides the contaminants carried over to the puparium from the feeding medium there may be a wax layer formed on the puparium. Pryor (1940) concluded that sclerotin formation made the cuticle 'lipophil'. He regarded the epicuticle as a simple protein later tanned and impregnated with lipides. It is suggested that the formation of sclerotin within the protein-lipide epicuticle of the larva of *Calliphora* during puparium formation leads to the exclusion of lipide on to the lipophil surface forming a distinct waxy layer. Abrasion of this layer by alumina dust or its disruption by detergents might be expected to result in an increase in water loss. This process of exclusion of waxy substances from within the epicuticle on to its surface would continue as long as the process of hardening and darkening occurs. The puparium is not fully hardened until pupation.

The prepupal cuticle does not control water loss in the early puparium (table 1, E). At pupation, occurring 25 hours after puparium formation, water

loss is efficiently controlled by the waxy layer of the delicate pupal cuticle (table 1, F). This has been extensively studied by Beament (1945).

The pore canals

The pore canals in newly moulted third instar *Calliphora* larvae appear as cytoplasmic filaments extending as far as the inner epicuticle. The deposition of endocuticle during the third instar results in the retraction of the cytoplasmic part of the filament; the outer non-cytoplasmic portion then becomes extremely difficult to distinguish from the surrounding endocuticle by usual staining procedures. Sections of cuticle, however, treated with 2 per cent. osmic acid show the pore canals very clearly. This observation suggests that during the retraction of the cytoplasmic filaments from the inner epicuticle a little lipidal material is left in the pore canals. Sudanophil material is also present particularly in the branching filaments just beneath the inner epicuticle. Pore canals branching fan-like within the inner epicuticle have been observed by Plotnikow (1904) in *Bombyx*, Dennell (1946) in *Sarcophaga*, and Way (1950) in *Diataraxia*.

Fresh sections of mature larval cuticle when examined either with transmitted light or under phase contrast show the pore canals clearly differentiated from the endocuticle. Phase contrast examination has revealed several interesting points about their structure in the mature cuticle (fig. 1, G). Two distinct regions of the canal are shown. The basal portion contains epidermal cytoplasm extending approximately one-third of the way through the endocuticle ($25\text{--}30\ \mu$). The distal portion shows what appear to be numerous fine granules within the laminae of the endocuticle and ends in the inner epicuticle. The distal portion of the canals does not show any lining and certainly does not look like a duct. The canal is not helicoidal but runs an almost straight course through the endocuticle. However, in young growing larvae, the pore canals are very irregular in their course through the endocuticle and appear as irregular wavy lines crossing the laminae of the endocuticle. A spiral or helicoidal course of the pore canals through the endocuticle sufficiently regular to ascribe a pitch to the helix as recorded by Dennell (1946) for *Sarcophaga* and Richards and Anderson (1942) for *Periplaneta* has not been observed.

One of the functions of the pore canals is the secretion of the inner epicuticle (Wolfe, 1954). Dennell (1946) reported the presence in *Sarcophaga falculata* of an endocuticular layer, the inner endocuticle, which contained no pore canals and was secreted in the mature larva just before puparium formation. This inner endocuticle was not found in *Calliphora*. Pore canals were found in osmic acid Orcein stained preparations connected to the epidermis right up to the commencement of browning of the puparium (fig. 1, F).

Larvae at the 'crop full' stage when immersed in ammoniacal silver nitrate solution for long periods (25–30 hours) showed series of distinct spots of silver within the epicuticle (fig. 1, H). These deposits corresponded to the pore canals in the inner epicuticle. They are not shown in larvae immersed

for short periods. The solution must penetrate through the outer epicuticle to the tips of the pore canals before being reduced. The outer epicuticle is slightly permeable to aqueous solutions in the mature larva. The van Wisse-lingh chitin test was performed on isolated pieces of cuticle which were mounted and examined in surface view. The pore canals showed up as dark purple dots on a paler purple background, and were found to be more concentrated in the lateral than the dorsal or ventral regions; approximately 17,400/sq. mm. in the lateral regions and 5,600/sq. mm. in the mid-dorsal and ventral regions. Fresh, unstained, transverse sections of the cuticle showed the endocuticle laminae crossed by many more lines in the lateral regions than elsewhere.

The endocuticle increases in thickness during the growth of the third instar larva, reaching a maximum of 80–85 μ . The laminae of the endocuticle are at all stages of growth penetrated by cytoplasmic extensions of the epidermis which continue distally as chitinized filaments into the inner epicuticle (fig. 1, G). An examination of the pore canals of fresh sections of the mature cuticle under phase contrast did not show any space between pore canal contents and surrounding endocuticle that might suggest plugs or cords of chitin within the pore canal lumen. Chitinous filaments were not found projecting from teased laminae of the outer region of the endocuticle. However, in young larvae, filaments were found projecting from endocuticular laminae in certain sections that had become teased apart during section cutting. These filaments were pieces of the cytoplasmic part of the pore canals which in the newly moulted larva extend up to the inner epicuticle (Wolfe, 1954). As the larva grows and the endocuticle thickness rapidly increases these cytoplasmic filaments are retracted. A marked differentiation still remains in the newly secreted endocuticle connecting the cytoplasmic portion of the pore canals to the inner epicuticle. It is this distal portion of the pore canals that gives a strong chitin reaction as shown by Dennell (1946). The cytoplasmic portion did not give a chitin reaction. This may be the reason why Dennell was unable to find pore canals in the inner region of the endocuticle in the mature larva.

The observations made above indicate that the pore canals remain connected to the epidermis throughout the third stadium. Way (1950) has shown in *Diataraxia* that in the soft cuticle the pore canals function only during the early stages of development and are then cut off from the epidermis by the development of an inner endocuticle. In areas of hard cuticle there was a thick heavily tanned exocuticle that continued to develop throughout the stadium and required the maintenance of the pore canal system from the epidermis. In *Calliphora*, however, the darkening and hardening processes occur at the end of the third stadium when the cuticle has reached its maximum thickness. During the period immediately before puparium formation polyphenols, basic protein, and enzymes accumulate in the inner epicuticle. It appears necessary that the pore canal conducting system be maintained between epidermis and inner epicuticle until puparium formation.

The pore canals of *Calliphora*, it is suggested, should not be regarded as distinct ducts or canals in the cuticle. Rather, they are thought to be differentiated regions through the laminae of the endocuticle possessing a greater porosity and able to transport materials necessary for the formation of the puparium quickly and selectively to the inner epicuticle.

The oenocytes at puparium formation

The oenocytes exhibit a secretory cycle at moulting and at puparium formation. During the period when the larvae are migrating away from the feeding medium, the amount of secretory granules within the oenocyte cytoplasm increases rapidly and reaches a maximum at the time of contraction of the cuticle to form the white puparium and then decreases during the first 5 hours of the puparium. The secretory cycle was followed by examining, under phase contrast, fresh oenocyte groups dissected from the larvae and puparium at different times during the third stadium (fig. 1, 1-N). The staining properties of the oenocyte secretion elaborated before moulting and puparium formation are those of an acidic, unsaturated lipide in association with protein. The larval oenocytes are completely histolysed by the 25th hour after the white puparium stage.

The role of the oenocytes in puparium formation is very obscure. None of the changes leading to the formation of the puparial cuticle seem to be correlated with oenocyte activity. It might be suggested that the oenocytes are associated with the production of a component necessary for the darkening and hardening of the puparium. But the phenolic precursors for this process are present in the inner epicuticle before secretion appears in the oenocyte cytoplasm. Moreover, histochemical tests on isolated oenocytes for phenols, oxidases, and dehydrogenases were negative. However, a suggestive correlation exists between the secretory activity of the oenocytes and the secretion of the prepupal cuticle during the first 6 hours after the white puparium stage. At moulting oenocyte secretive activity follows strikingly the deposition of the protein-lipide epicuticle (Wigglesworth, 1948; Wolfe, 1954).

The secretion granules within the oenocyte cytoplasm as well as the prepupal cuticle are stained by Sudan black. The peak in the secretive activity of the oenocytes occurs just before the prepupal cuticle is formed. Immediately after the secretion of the prepupal cuticle histolysis commences in the epidermal cells and oenocytes, which separate from the cuticle and become replaced by bands of actively dividing imaginal epidermal cells spreading over the larval epidermal cells displacing them into the interior of the puparium.

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The Identification of Thyrotrophin-secreting Cells in the Pituitary Gland of the Minnow (*Phoxinus phoxinus*)

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With one plate (fig. 2)

SUMMARY

Evidence is given for the existence of two main types of cyanophil cell in the median zone of the glandular lobe (adenohypophysis) of the minnow, distinguishable by their distribution and by their cytological characteristics. Both types are positive to the periodic acid Schiff (PAS) technique, but one (type 2 of this account) also gives a positive response to the aldehyde-fuchsin (AF) technique of Gomori, as used by Halmi and by Purves and Griesbach in studies of the mammalian pituitary. In fish which have been immersed in thiouracil solution the type 2 cells show degranulation and vacuolation, and their characteristic positive AF response is very greatly weakened or lost. For these reasons the type 2 cells are believed to be responsible for the secretion of thyrotrophin, and appear to be very closely comparable with the thyrotroph cells of the pituitary of the rat.

INTRODUCTION

IN drawing attention in previous papers to the effect of thiourea in delaying the sexual maturity of the minnow and to the existence of seasonal variation in the heights of the epithelial cells of its thyroid gland (Barrington and Matty, 1952, 1954), we have emphasized the difficulty of interpreting these facts in the absence of adequate information regarding the histophysiology of the pituitary gland of this animal. It has therefore been our purpose in the present work to attempt a demonstration of the source of thyrotrophic hormone (TSH) by employing thiouracil to promote an increased output of the hormone and examining the effect of this upon the pituitary gland, using as histological techniques certain procedures which have recently been applied to this problem in the rat (Purves and Griesbach, 1951). During the course of this study Atz (1953) has published the results of a similar investigation into the pituitary gland of another Teleost, *Astyanax*. As will be seen, our own results, obtained independently of hers, appear to be in broad agreement with them, but the situation in *Phoxinus* appears to be even more clear-cut, not only because of the spatial localization of the presumed thyrotroph cells in this fish, but because we have been able to demonstrate a differential response of these to the Gomori aldehyde-fuchsin stain similar to that obtained by Purves and Griesbach in the pituitary of the rat.

METHODS

Minnows (*Phoxinus phoxinus* L.) were obtained in October from the Fresh-water Biological Association, Windermere, some being used without experimentation while others were placed in groups of about 20 in tanks measuring

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14 in. \times 9 in. \times 9 in. The temperature was controlled at $18^{\circ} \pm 1^{\circ}$ C. and, since it was intended also to study the sexual condition of the animals, they were subjected to additional artificial illumination (Bullough, 1940), the intensity of this at the bottom of the tanks being about 130 foot-candles. The results of this illumination have no immediate bearing upon the present report and will not be described here, but it may be mentioned that it was increased by 1 hour per day, starting with 8 hours per day, up to a full 24 hours per day of artificial light. Some of the tanks contained plain tap-water, others a solution of 0.03 per cent. thiouracil. The water was well aerated and was changed every 3 days, the fish being fed just previously to this with an ample supply of blowfly larvae supplemented by minced horse-flesh.

Males only were selected for study, and were killed by pithing; the lower jaw and cranium were then fixed separately in mercuric-formaldehyde, the roof of the buccal cavity being first partly removed in order to ensure good fixation of the pituitary without disturbing it. Sections were cut at 5μ in the usual way after wax embedding, and were stained by the Azan method of Heidenhain, the periodic acid Schiff (PAS) method (with or without counter-staining by orange G, Pearse, 1953), and the aldehyde-fuchsin (AF) method of Gomori, as used by Halmi (1952). The significance of these methods in their application to the pituitary of the rat is that the aniline blue of the Azan method reveals the cyanophil cells (the so-called 'basophils' of many earlier authors); the PAS method also stains these cells, and is thus held to indicate them as the probable source of the glycoprotein hormones FSH, LH, and TSH (Pearse, 1952), while the AF method, which stains some, but not all, of the PAS-positive cells has been considered (Purves and Griesbach, 1951) to indicate the thyrotroph cells. It should be made clear, however, that while the chemical basis of the PAS method is well established, the AF method is at present purely empirical (Halmi and Davies, 1953), and the conclusion that the AF-positive cells are thyrotrophs is based upon the observed responses of these cells to thyroidectomy, with some support from a study of conditions in the young animal (Siperstein and others, 1954). We find, as did the latter authors, that the AF method is capricious, and demands careful control of the ripening of the staining solution; we have therefore been careful throughout to check the reaction by staining standard test slides of the pituitary of the minnow and the rat, so that negative results are known to be significant and not to result from inactivity of the reagent, while slides of material from control and goitrogen-treated animals have been taken through the technique side by side.

It is an obvious advantage, when comparing the response of cells to the PAS and AF methods, to be able to stain the same section consecutively by the two techniques. The procedure adopted to this end by Purves and Griesbach in their study of the rat pituitary was to superimpose the PAS coloration upon the AF, the two being distinguished by careful control of the intensity of the staining and of the photomicrographic technique. In the present work it has been found more convenient to bleach the section by

immersion in very dilute 'Milton' after study of the AF reaction, and then to apply the PAS technique. The advantage of this procedure is that it is possible, by examination of the section after the bleaching stage, to establish the complete elimination of the AF coloration, and in this way to guard against the possibility of a false positive response being given in the PAS preparation as a result of the persistence in it of the original AF reaction.

OBSERVATIONS

Normal cytology

The general structure of the pituitary gland of teleosts, not easily comparable in some respects with that of mammals, has been described and discussed by a number of authors, notably by Kerr (1942) and by Green

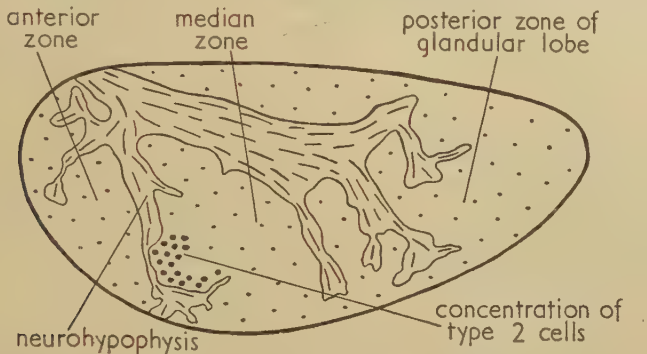


FIG. 1. Diagram of a longitudinal section of the pituitary gland of a minnow taken just lateral to the stalk. The position of the concentrations of type 2 cells varies from section to section, and extends dorsally as well as posteriorly from the area indicated.

(1951). It will not, therefore, be dealt with here in detail, and it will be sufficient to say that in the minnow it is composed of two major components which are closely interlocked (fig. 1). The predominantly more ventral of these forms the 'glandular lobe', essentially equivalent to the adenohypophysis of other vertebrates; this is differentiated by its cytological characteristics into three zones, of which the most posterior has been compared with the pars intermedia of the mammalian gland (Green, 1951), the median ('transitional lobe' of some authors) with the pars distalis, and the anterior with the pars tuberalis (Atz, 1953). The second, more dorsal, component may be regarded as the neurohypophysis, and this ramifies by characteristic finger-like outgrowths into the glandular lobe, carrying blood-vessels with it.

Of the various cell-types distinguishable in this gland, the present work is concerned only with those cyanophils which (together with acidophils) are a conspicuous feature of the median zone, and which are also seen, but to a smaller extent, in the anterior zone. The number and size of these cyanophils vary considerably with the seasonal and experimental conditions, the teleost gland being very labile, as Green points out, but in the present material it has been possible to distinguish two main categories. First, there are cells

(type 1, fig. 2, A) with deeply cyanophil contents, the nature of which is not clearly defined in Azan preparations, but which in general have a flocculated or alveolated appearance. The nuclei are often pressed to one side of the cell, stain rather heavily, and may be somewhat flattened, crescent-shaped, or contorted in outline. These cells vary in size, and are undoubtedly enlarged in animals which have been artificially illuminated and in which the reproductive system has been activated. For this reason, and having regard to observations on other fish (see, for example, Kerr, 1948), they are here provisionally regarded as gonadotrophs, the fact that they are PAS-positive (see below) being in accordance with this view.

Contrasting with these is another type (type 2, fig. 2, A), occurring in conspicuous groups in a very characteristic position (fig. 1); as a result of this localization they can readily be distinguished in serial sections, varying in number and position from section to section, but usually lying close against the neurohypophyseal tissue, either along the dorso-ventral boundary between the anterior and median zones, or farther back within the latter. These cells, which, for reasons to be explained below, are here regarded as thyrotrophs, are also distinguishable by cytological characteristics. They stain more lightly with aniline blue than do the type 1 cells, and their contents appear more tenuous and even less well-defined. Their nuclei (fig. 2, A) are large and rounded, and in these respects, as in their weaker chromophilia, contrast with those of the other cyanophils. It is not suggested that these cells are only found in conspicuous groups; indeed, individual cells with similar features are certainly to be seen scattered in other parts of the median zone, but their concentration in the manner described is a striking characteristic of them.

The probability that both types of cyanophil are involved in the secretion of glycoprotein hormones is indicated by the fact that they both give a positive reaction in PAS preparations (see above, p. 194), in which they are

FIG. 2 (plate). A to G, pituitary gland of minnow; H and I, thyroid gland of minnow.

A, type 1 cells lie at the lower border, and a type 2 cell is in focus at the top centre, immediately above a clear strip of neurohypophyseal tissue. A smaller cyanophil (see text) lies just below the latter to the right of centre. (Azan preparation.)

B, PAS preparation showing type 1 cells below and type 2 cells above; both are PAS-positive.

C, vacuolation of type 2 cells in an animal which had been immersed for 94 days in thiouracil solution. (Azan preparation.)

D, AF preparation, showing the positive response of type 2 cells.

E, PAS preparation of the same field as in D, showing the PAS-positive response of type 2 cells.

F, AF preparation. A strong positive response is shown in the type 2 cells, which extend in a curved line from the top left corner. Type 1 cells, situated centrally, are negative and are only faintly distinguishable.

G, PAS preparation of the same field as in F. Only a very light reaction has been evoked (compare fig. 2, B which illustrates a much stronger one), but the type 1 cells are now clearly indicated in the centre.

H, thyroid gland of control minnow, showing normal follicles.

I, thyroid gland of a minnow which had been immersed for 94 days in thiouracil solution. The type 2 cells of the pituitary of this fish are shown in fig. 2, C.

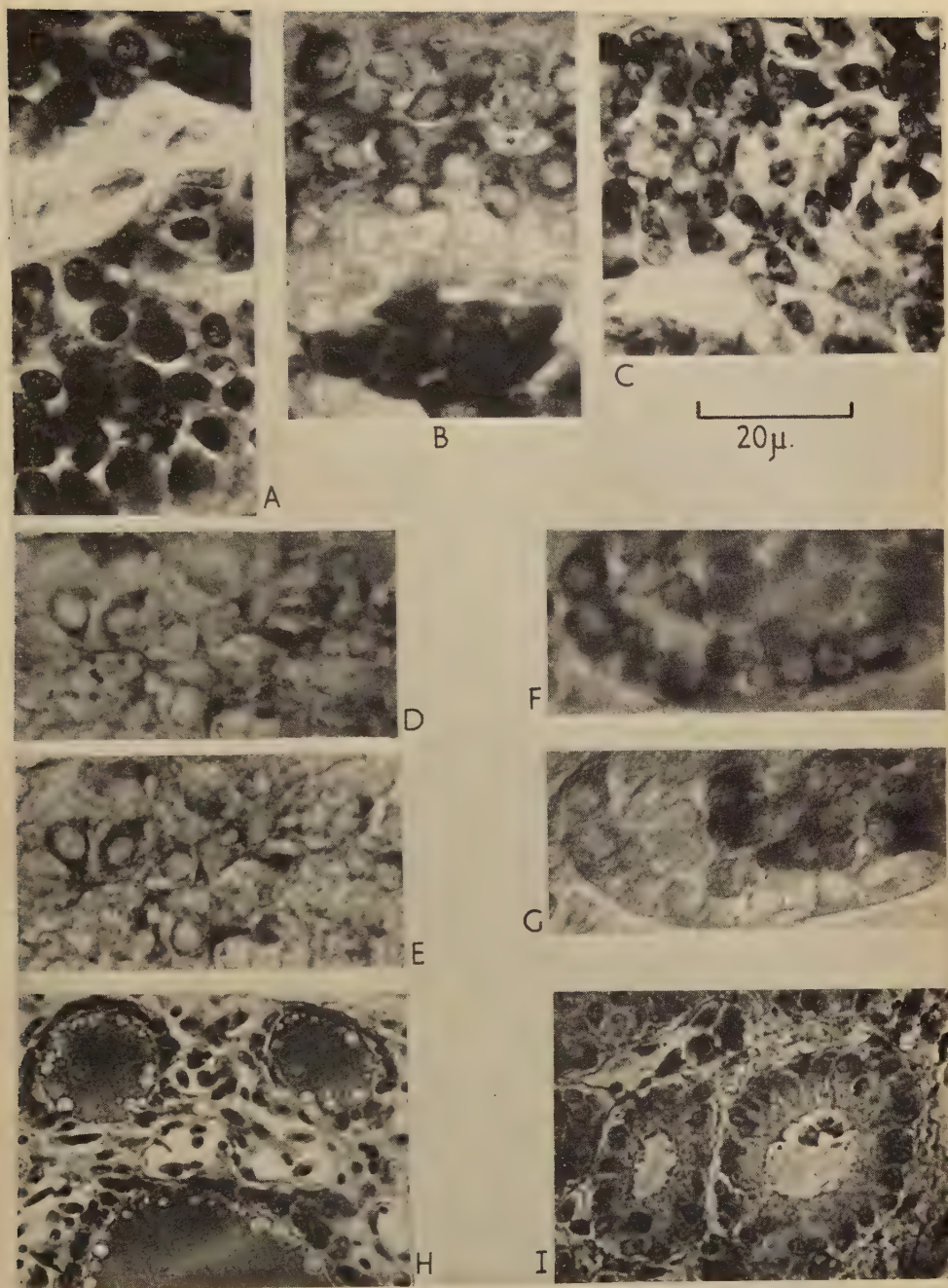


FIG. 2
E. J. W. BARRINGTON and A. J. MATTY

distinguishable from each other by their distribution and nuclear characteristics, the use of the orange G counterstain serving to differentiate both of them from the other cells present. The type 2 cells (fig. 2, B) show a fine magenta-coloured reticulum or granulation, which may form a fringe closely embracing the nucleus. The type 1 cells (fig. 2, B) show a similar coloration, but many of them, usually the larger, are also distinctly acidophilic in appearance as a result of the existence in their cytoplasm of granules or droplets which take up the orange G. Comparison with Azan preparations makes it clear that in these the acidophil inclusions are almost completely unstained, their positions being represented by the clear spaces amongst the blue-stained flocculi or reticulum. They seem to be particularly characteristic of the type 1 cells, but the possibility that they may occasionally be present in type 2 cells has not been entirely excluded.

The use of the AF technique produces an interesting cytological picture in which the nerve fibres of the tractus hypothalamo-hypophyseos, and the reticulum to which they give rise in the neurohypophysis, are stained an intense purple; it would therefore appear that this method provides another means of revealing the presumed neurosecretory material which has recently been effectively demonstrated by chromium haematoxylin (Bargmann and Hild, 1949). In *Astyanax* (Atz, 1953) this method apparently colours all the cyanophils in the median zone, but in the minnow it differentiates the type 2 from the type 1, the contents of the former giving a delicate but well-defined purple coloration while the latter are almost or completely unstained. The close correspondence, amounting to virtual identity, between the AF-positive and PAS-positive material of the type 2 cells is illustrated by a comparison of figs. 2, D and 2, E, which show the same field of these cells stained first by the former technique (fig. 2, D), and then, after bleaching, by the latter (fig. 2, E). If the section is coloured relatively lightly with the AF procedure (using an orange G counterstain), the type 2 cells alone take a distinctive purple colour, the type 1 appearing greyish or greyish-brown by contrast. If a stronger reaction is obtained by prolonging the time of treatment, the purple colour may be taken up slightly by the type 1 cells, but the reaction of the type 2 remains distinctive by its brightness and clarity. It can, therefore, safely be said that this latter type is distinguishable from type 1 by its strongly positive AF reaction, and this difference in the behaviour of the two types may be seen by comparing figs. 2, F and 2, G. The former shows a field coloured by the AF technique, with type 2 cells sharply indicated and type 1 only faintly visible. Fig. 2, G shows the same field after bleaching and re-staining with the PAS technique; the reaction here is a weak one, the type 2 cells being only very lightly coloured, as may be judged by comparing them with the more strongly coloured ones in fig. 2, B, but the type 1 cells are now clearly indicated in complete contrast to their virtually negative AF response.

There is much variation in the size of the cyanophils, dependent upon the physiological condition of the animal, but in particular there are smaller

cells which, in Azan preparations, are not easily assignable to either of the main two types. These often have a nucleus (fig. 2, A) which is contracted and densely chromophil, and may be contorted or deeply incised to such an extent as sometimes to make the cell appear multinucleate. Some of these grade into the type 1 cells, others can be seen to be AF-positive, but it is not clear whether they are all to be regarded as divisible into two types like the larger ones, or whether many of them are undifferentiated cells from which either type could arise. Some at least are probably resting cells from which the larger and active ones are recruited, others may be exhausted ones, and a final opinion on them is reserved until further work on the effect of illumination has been completed.

The effect of thiouracil

Reference has been made above to the view of Purves and Griesbach (1951) that those cells in the rat which are both PAS-positive and AF-positive are thyrotrophs, and that this is also true of the type 2 cells of the minnow is strongly suggested by the results of immersion of the fish in thiouracil solution.

Nine control fish, kept in plain water for 58 days, all had thyroids which were normal in appearance (fig. 2, H), with a low epithelium and with follicles well filled with colloid. In contrast to this, ten fish which had been treated with thiouracil (seven for 59 days and three for 94 days) were highly goitrous, showing enormous enlargement of the thyroid cells with a substantial and sometimes virtually complete loss of colloid (fig. 2, I), indicative, of course, of a heavy outpouring of TSH in response to the inhibitory action of the goitrogen on synthesis of the thyroid hormone.

Now Atz (1953) found that treatment of *Astyanax* with thiourea produced increased vacuolation and partial degranulation of the cells believed by her to be thyrotrophs, and in the minnow the thiouracil treatment can often be seen to produce a similar effect, although there is variation amongst individuals. Usually, however, it is easy to locate, in the expected areas, groups of type 2 cells, still to some extent cyanophilic in reaction, but showing varying degrees of vacuolation or breaking-down of their contents (fig. 2, C). Their appearance contrasts markedly with the homogeneity of the rest of the gland, which seems to be unaffected by this treatment, and certainly suggests that they may well be thyrotrophs, their condition resulting from their intense secretory activity promoted by the goitrogen treatment. The fact, however, that these cells, unlike the corresponding ones in *Astyanax*, can be differentiated from the type 1 cells by their AF response makes it possible to carry the analysis further by developing a more detailed comparison with the mammal.

Purves and Griesbach (1951) found that the AF reaction disappeared from the thyrotrophs of the rat after surgical thyroidectomy, a result which they ascribed to the increased output of TSH, the AF reaction being, presumably, in some way an indication of the presence of stored TSH which was lost

from the cells when the demand was increased by the removal of the circulating thyroid hormone. In order to provide a more exact comparison with the present study of the minnow, which has involved chemical and not surgical thyroidectomy, 20 mg. of thiouracil per day was administered orally to rats ranging in weight from 416 g. to 535 g. After 12 days of this treatment there was a marked weakening (although not a complete disappearance) of the AF reaction in the pituitary, accompanied by the expected goitrous reaction of the thyroid. It is thus reasonable to expect a reduction or elimination of the reaction in the AF-positive cells of the thiouracil-treated minnows if they are, in fact, thyrotrophs, and this is exactly what actually occurs, for the nine control animals, like others which had not been maintained in the laboratory at all, showed a clear positive AF reaction, while in the ten thiouracil-treated fish the reaction was very weak or absent.

The slight variation found here may be due in part to variations in the reactivity of the reagent, although since slides from a number of animals were always stained together in groups this is not likely to be very marked. More significant are the undoubted variations in the responses of individual fish to the goitrogen treatment, an extreme example of this being provided by an eleventh thiouracil-treated animal, which, despite having been immersed in the goitrogen for 59 days, differed from the others in having a much more normal thyroid, with abundant colloid and an epithelium only slightly increased in height. The significant feature of this fish was that it also differed from the others in having a clear positive AF reaction in the type 2 cells. Presumably it had proved relatively refractory to the goitrogen, the demand for TSH had remained small, and this was precisely mirrored in the persistence of the AF reaction. Another, although less extreme, example was encountered during the examination of the three fish which had been treated for 94 days with thiouracil. One of these, in contrast to the specimen illustrated in figs. 2, c and 1, showed type 2 cells which, despite some signs of vacuolation, retained a considerable amount of cyanophilia and some of which still gave a very faint positive AF response. The thyroid of this animal proved to retain a marked amount of colloid, despite the pronounced hypertrophy of its cells, so that again there was a correlation between the appearance of this gland and the condition of the type 2 cells, the latter being indicative of a less intense demand for TSH than had been experienced in some of the other fish.

Such facts suggest a very close similarity between conditions in the minnow and in the rat, the positive AF response being, in other words, an index of the presence of stored TSH and of a limited demand for this hormone. Further evidence for this was obtained by transferring some fish to plain water after 59 days of thiouracil treatment, and allowing them 32 days of recovery. At the end of this period three animals were selected at random for comparison with those which had received a full 94 days' treatment with the goitrogen. The latter, as already explained, had a highly goitrous thyroid, but the former showed a restitution of the normal histological appearance of

the gland, with a low epithelium and abundant colloid. Evidently the TSH demand had been greatly reduced in these during the recovery period, and on the view advanced above it would be expected that their type 2 cells might be AF-positive as a result of resumption of TSH storage. This was in fact the result actually obtained, the pituitaries of all three showing a rather slight but quite definite positive reaction.

DISCUSSION

The results recorded here are based upon a study of 28 male fish (11 thiouracil-treated, 3 thiouracil-treated with a period of recovery ensuing, and 9 controls, together with 5 which had received no laboratory treatment at all), and are uniformly consistent with the view that two types of cyanophil can be distinguished in the median zone of the glandular lobe of the pituitary, and that TSH secretion is the property of that type which gives a positive AF response and which is found in particular abundance at the boundary between the anterior and median zones, although it is probably not exclusively localized there. The two types can also be distinguished by their nuclear characteristics, and in this respect appear to correspond with the two types described by Atz (1953) in *Astyanax*, in which genus the supposed thyrotrophs have usually a round or oval nucleus, while the supposed gonadotrophs (type 1 of the present account) have nuclei which, while frequently round or oval, may also be sharply indented, bean-shaped, crescent-shaped, or twisted, as is very often the case in *Phoxinus*. Both types are stained by the AF reaction in *Astyanax*, and in this respect, as in their distribution within the gland, the situation in the minnow is different, but a study of a wide range of genera is required before any useful comment can be made on such differences between members of a group as large and as variable as the Teleostei. It has been mentioned above (p. 198) that the smallest cyanophils cannot be as certainly divided into two categories as can the larger ones, and, as Atz (1953) has remarked, the limits of variability of the cell types and the degree of ultimate discontinuity between them are as yet uncertain. Much further work is clearly needed to elucidate the mechanism by which fluctuations in pituitary activity in these fish are effected, and to determine how far one type is recruited at the expense of others.

There has been little reference in studies of the pituitaries of other teleosts to differentiation of the cyanophils. Oliveureau (1954) has observed signs of activity in these cells in the salmon at periods of thyroid hyperactivity, and has concluded that they are indicative of TSH secretion, but she does not refer to any specific differentiation of thyrotrophs and gonadotrophs. Kerr (1942), on the other hand, has given purely cytological criteria for the existence of two types of cyanophil in the perch, and it will clearly be of interest to determine whether such cells can be further characterized by the techniques described here. Evidence is also needed as to the source of ACTH, but in the meantime the results of the present work provide a basis for a more critical

analysis of the reactions of the pituitary gland of the minnow to experimental or seasonal conditions than has previously been possible. Only when such an analysis has been made for a particular species does it become possible to determine whether the rate of secretion of the various glycoprotein hormones of the pituitary is linked in any way, or whether they are entirely independent of each other and influenced by independent external factors. This is an issue of importance, having regard to the evidence for the existence of seasonal variation in thyroid activity in teleosts (Barrington and Matty, 1954), and it is hoped to present later some observations on this aspect of the problem.

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Cytological Fixation by Chromic Acid and Dichromates

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With one plate (fig. 4)

SUMMARY

0.07 *M* (1 per cent.), 0.17 *M* (2.5 per cent.), and 0.34 *M* (5 per cent.) solutions of analytical-grade potassium dichromate have pH values of 4.10, 4.05, and 3.85 respectively. The values for corresponding solutions of chromic acid are 1.20, 0.85, and 0.70. The oxidation potential for potassium dichromate is 0.76 V, and for chromic acid, 1.10 V.

During fixation for 18–20 hours at room temperature, with either reagent, there is a small decrease in hydrogen-ion concentration but no appreciable change in oxidation potential. During postchroming for 48 hours at 37° C., there is a further decrease in hydrogen-ion concentration, but only in the case of chromic acid is there a decrease in oxidation potential.

The morphological features of the fixed cells are determined almost entirely by the fixing reagent. Postchroming can influence staining properties. There are two characteristic fixation 'pictures' depending upon the pH of the reagent: (1) with chromic acid and the more acidic dichromates (barium, calcium, mercuric, or silver) there is destruction of the mitochondria and disorganization of the cytoplasm and nuclear contents, resisted only by the nucleolus, and (2) with potassium dichromate and the other less acidic dichromates (ammonium, lithium, or sodium), the mitochondria, cytoplasm, and nucleus are well fixed. With various tissues from mice, the transition between the two types occurs around pH 3.4–3.8.

The chemical mechanism of cytological fixation by anionic chromium reagents remains to be elucidated. Oxidative reactions do occur. Probably more important are ionic interactions and complex formations, often with associated precipitation, involving various tissue constituents and the several chromium ions.

INTRODUCTION

FIXATION by various chemical reagents is still the commonest method of initially preparing tissues or cells for microscopical examination, whether morphological or histochemical. Usually, several substances are combined in solutions of empirical composition. A knowledge of the properties and cytological effects of each component is essential to understanding the action of such mixtures and to assessing the results obtained with them (Baker, 1950). Nevertheless, this is one of the least investigated, although most fundamental, aspects of cytological technique.

Chromic acid and potassium dichromate have been widely used in fixing mixtures since their respective introductions by Hannover in 1840 and Müller

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in 1859. Fixation by these reagents, especially their effects on nuclei, was studied by Flemming (1880) and by Burchardt (1897). Most comprehensive are the contributions of Zirkle (1928, *a* and *b*) who worked with *Zea mays*. The present investigation has concerned the hydrogen-ion concentrations and oxidation potentials of solutions containing chromic acid, potassium dichromate, or both, and the effects of these reagents upon various mammalian tissues. Additional observations regarding seven other dichromates are described.

The general properties of chromic acid and its salts are dealt with in the various treatises on inorganic chemistry. The earlier literature has been comprehensively reviewed by Koppel (1921) and by Mellors (1931). The use of chromic acid and dichromates as fixing agents is mentioned in most texts on cytological or histological technique. It has been discussed in greater detail by Berg (1926) and by Baker (1950). In so far as tissue fixation by solutions containing these substances is concerned, ionic interactions, the formation of complexes, and oxidative processes are the most important. These are influenced by the hydrogen-ion concentration of the reaction medium.

Few data have been published concerning the hydrogen-ion concentrations of various fixing solutions. Yamaha (1925) did study several mixtures. A wider variety was investigated by Petrunkevitch and Pickford (1936), who used the glass electrode. Apart from Zirkle (1928, *a* and *b*), who estimated the pH of his various chromic acid and dichromate solutions colorimetrically, Lassek and Lunetta (1950a) are among the few who have recognized that 'basic to an understanding of the role of hydrogen-ion concentration in fixation of tissues is a knowledge of the pH values of the chemicals which are commonly employed in making the numerous fixing mixtures'. These investigators used the glass electrode to measure the hydrogen-ion concentrations of 21 primary fixing solutions, including 1 per cent. chromic acid and 3 per cent. potassium dichromate.

Ionization

The ionization of chromic acid and its salts is discussed by Ricci (1952). Chromic acid is a dibasic acid having, in addition to the divalent chromate ion, CrO_4^{2-} , a univalent anion, the hydrochromate ion, HCrO_4^- , which condenses to form polyacids, especially dichromate, $\text{Cr}_2\text{O}_7^{2-}$, in aqueous solutions. Insignificant amounts of tri- and tetrachromates are also formed. The ionizing, molecular chromic acid in a solution prepared by dissolving chromium trioxide in water is sometimes represented as H_2CrO_4 and sometimes as $\text{H}_2\text{Cr}_2\text{O}_7$. Which of these is the original solute cannot be determined from the equilibrium relations of the solution. The distribution of the various charged and uncharged species and their behaviour with respect to the hydrogen-ion concentration and to the total amount of solute, however, are independent of the formula chosen for 'chromic acid'. For the purposes of this discussion, H_2CrO_4 has been adopted.

The species in an aqueous solution of this acid are H_2CrO_4 , HCrO_4^- , $\text{CrO}_4^{=}$, and $\text{Cr}_2\text{O}_7^{=}$, for which the following equilibria exist:

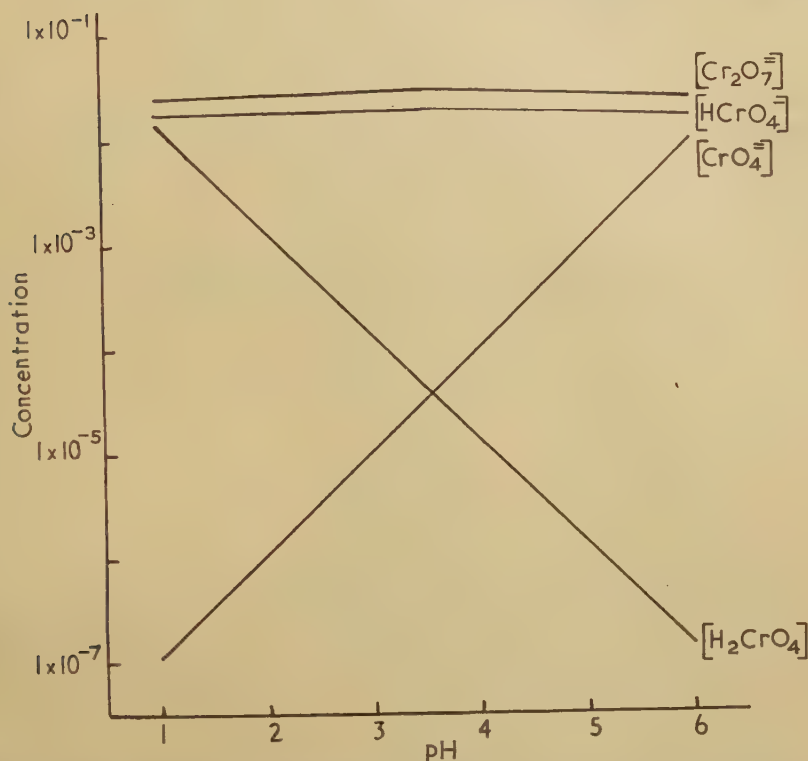


FIG. 1. Relation between the concentrations (logarithmic scale) of chromium species and the pH of 0.17 *M* solutions of chromic acid and its salts. (Based on calculations.)

These various species are related by the usual ionization constants:

$$K_1 = \frac{[\text{H}^+][\text{HCrO}_4^-]}{[\text{H}_2\text{CrO}_4]} = 0.16, \quad (1)$$

$$K_2 = \frac{[\text{H}^+][\text{CrO}_4^{=}] }{[\text{HCrO}_4^-]} = 3.2 \times 10^{-7}, \quad (2)$$

and the complex constant,

$$K = \frac{[\text{Cr}_2\text{O}_7^{=}] }{[\text{HCrO}_4^-]^2} = \frac{1}{0.023} \approx 43.5. \quad (3)$$

The numerical values given are those determined by Neuss and Riemann (1934), who have discussed earlier estimates. As equation (3) indicates, the

ratio of the concentrations of dichromate and hydrochromate ions is constant and independent of the concentration of hydrogen ions. The distribution and concentrations of all four chromium species, however, are determined by the hydrogen-ion concentration of the solution and by the analytical concentration of the dissolved chromium trioxide (chromic anhydride). If M represents the latter, then

$$M = [\text{H}_2\text{CrO}_4] + [\text{HCrO}_4^-] + [\text{CrO}_4^{=}] + 2[\text{Cr}_2\text{O}_7^{=}], \quad (4)$$

and the replacement of part of the chromic acid by an equivalent amount of a chromate or a dichromate will have the effect of merely adding a base; that is, of modifying the hydrogen-ion concentration.

From equations (1)–(4), the following relationships can be derived:

$$\begin{aligned} [\text{HCrO}_4^-] &= - \left[\frac{K_1 K_2 + K_1 [\text{H}^+] + [\text{H}^+]^2}{4KK_1[\text{H}^+]} \right] + \sqrt{\left(\frac{K_1 K_2 - K_1 [\text{H}^+] + [\text{H}^+]^2}{4KK_1[\text{H}^+]} \right)^2 - \frac{M}{2K}}, \\ &\quad (5) \end{aligned}$$

$$[\text{H}_2\text{CrO}_4] = \frac{[\text{H}^+][\text{HCrO}_4^-]}{K_1}, \quad (6)$$

$$[\text{CrO}_4^{=}] = \frac{K_2[\text{HCrO}_4^-]}{[\text{H}^+]}, \quad (7)$$

$$[\text{Cr}_2\text{O}_7^{=}] = K[\text{HCrO}_4^-]^2. \quad (8)$$

Differentiation of these equations with respect to the hydrogen-ion concentration provides additional indications of the behaviour of the various species in a chromic acid or dichromate solution. The amount of undissociated acid increases with increasing hydrogen-ion concentration: $d[\text{H}_2\text{CrO}_4]/d[\text{H}^+]$ is positive. The concentration of chromate ion decreases with increasing hydrogen-ion concentration: $d[\text{CrO}_4^{=}]/d[\text{H}^+]$ is negative. The concentrations of both the hydrochromate and dichromate ions pass through maxima at $\text{H}^+ = \sqrt{(K_1 K_2)}$. The behaviour of each species over the range pH 1.0–pH 6.0 is represented in fig. 1, which was prepared from the values calculated for equations (5)–(8) by using $M=0.17$.

Previously, it has been suggested that the morphological differences between cells fixed in chromic acid and those fixed in potassium dichromate might be attributable to a large difference between the proportions of the principal chromium anions, $\text{Cr}_2\text{O}_7^{=}$ and HCrO_4^- , in the two reagents. In reality, the ratio of the dichromate-ion concentration to the square of the hydrochromate-ion concentration remains constant (the complex constant). The two solutions are compared in fig. 2.

Oxidation

Although oxidation, or reduction, occurs during the fixation of tissues by many mixtures, apparently no one has investigated the redox potentials of fixing solutions. Relevant data for the reactions in which most of the important

primary reagents participate can be found in the physicochemical literature, especially in the monographs by Conway (1952) and by Latimer (1952). Certain aspects of oxidation by dichromate are discussed by Kolthoff and Furman (1931). More recent studies have been reviewed by Furman (1942).

The standard potential, E^0 , for oxidation by dichromate,

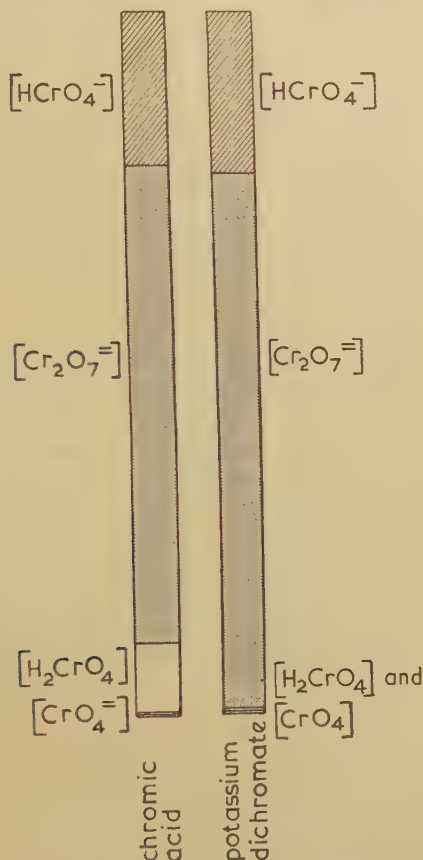
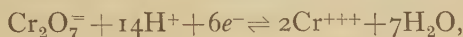


FIG. 2. Comparison of 0.17 *M* solutions of chromic acid and potassium dichromate. For chromic acid, $[\text{HCrO}_4^-] = 0.037$; $[\text{Cr}_2\text{O}_7^{2-}] = 0.058$; $[\text{CrO}_4^{2-}] = 1.2 \times 10^{-7}$; $[\text{H}_2\text{CrO}_4] = 0.020$; $K = 43$. For potassium dichromate, $[\text{HCrO}_4^-] = 0.039$; $[\text{Cr}_2\text{O}_7^{2-}] = 0.066$; $[\text{CrO}_4^{2-}] = 1.2 \times 10^{-4}$; $[\text{H}_2\text{CrO}_4] = 2.2 \times 10^{-5}$; $K = 43$. Arithmetic scale. (Based on calculations. The very low concentrations could not be accurately represented in the diagrams.)

has been recalculated from thermodynamic data by Latimer (1952) to be 1.33 volts. (The convention of making the potential of this and similar reactions positive (Hewitt, 1950; Conway, 1952) will be observed here.) The large influence of the hydrogen-ion concentration upon the oxidation potential is apparent from the equation,

$$E = E^0 - \frac{0.059}{6} \log \frac{[\text{Cr}_2\text{O}_7^{2-}][\text{H}^+]^{14}}{[\text{Cr}^{+++}]}$$

If the ratio of dichromate to chromic-ion concentrations were kept constant, a change in electrode potential of approximately 0.135 volt would be expected theoretically for a tenfold change in hydrogen-ion concentration. In reality, however, the change in potential per unit change in pH is usually considerably less. It is not constant and is influenced by the prevailing hydrogen-ion concentration and the nature of any other electrolytes present (Kolthoff and Furman, 1931).

Fixation

The fixation of plant tissues by solutions of chromic acid and some thirty dichromates was studied in detail by Zirkle (1928, *a* and *b*). He adopted 2.5 per cent. aqueous chromium trioxide as the basis for his experiments. Approximate pH values were estimated colorimetrically. Root tips of *Zea mays* were fixed for 36–40 hours and stained with Heidenhain's iron haematoxylin. Zirkle found that

the fixation image of a dichromate depends upon the pH at which it is used. If the solution is more acid than a given critical point, the image will be practically that of chromic acid, i.e. in resting cells the nucleolus will be a darkly staining globule in the centre of a hollow nucleus whose periphery is formed by a chromatin reticulum. No mitochondria will be preserved and the cytoplasm will be disorganized. In dividing cells, the chromosomes and spindle fibers will be well fixed. If the solution is on the alkaline side of the critical point, the fixation image will be quite different. In the resting stage the nucleolus is fixed as in the acid fixative, but here it appears in a solid nucleus composed of fixed nuclear lymph. The chromatin and spindle fibers will be dissolved so the tissue will show no mitotic figures. The mitochondria and cytoplasm will be well fixed. The change from one fixation image to the other is as a rule sudden and complete, the point of change depending upon the dichromate used . . .

and ranging from pH 4.2 to pH 5.2. Zirkle concluded that his findings could be explained best on the basis of reactions between the ionic species of the fixing reagents and the charged groups of the tissue components, accompanied by reduction of some of the dichromate so that ultimately both anionic and cationic chromium are present.

Most of the investigations to be described here have concerned aqueous solutions of chromic acid and potassium dichromate because these are the chromium compounds most frequently used in fixing mixtures. The initial problem was to ascertain the influence of hydrogen-ion concentration upon the fixation of tissues from mice by these reagents; that is, to find whether Zirkle's observations also applied to various mammalian cells. The studies were extended to include the oxidation potentials of the solutions and fixation by other dichromates. Particular attention was given to the preservation of mitochondria.

Potassium dichromate was chosen as the basis for all experiments. It was studied over the range of concentrations in which it usually occurs in fixing

mixtures, 1–5 per cent. Such solutions are from 0.07 to 0.34 molar with respect to chromium trioxide. Chromic acid, therefore, was studied over this range of concentration. Solutions of intermediate hydrogen-ion concentrations were prepared by mixing the potassium dichromate and chromic acid reagents in varying proportions. For higher pH values, mixtures of potassium dichromate and potassium chromate were used. Solutions of ammonium, barium, calcium, lithium, mercuric, silver, and sodium dichromates were studied in less detail.

Throughout the following account of experimental studies, the molarity of solutions containing chromic acid or its salts is expressed in terms of their contents of chromium trioxide, designated by M , rather than of their content of the solute, M . For example, 5 per cent. potassium dichromate, although only 0.17 M with respect to potassium dichromate, is 0.34 M in terms of chromium trioxide. All solutions which are 0.34 M , therefore, are comparable with 5 per cent. potassium dichromate; 0.17 M solutions, with 2.5 per cent., and 0.07 M , with 1 per cent. Percentage compositions are expressed as grams of solute per 100 ml. of solution.

MATERIALS AND METHODS

The 0.34 M stock solutions of analytical grade reagents were prepared by dissolving 50.00 g. of potassium dichromate, 65.96 g. of potassium chromate, or 34.00 g. of chromium trioxide in sufficient distilled water to give 1,000.0 ml. of solution. Lower concentrations, 0.17 M or 0.07 M , were obtained by diluting these stock solutions with distilled water, 1:1 or 1:4 respectively. For 0.001 M reagents, 0.75 ml. of a 0.34 M solution was mixed with 250 ml. of 0.85 per cent. aqueous sodium chloride.

Various mixtures were prepared such that from 0.15 to 80.0 per cent. of the solute was chromic acid, the remainder being potassium dichromate. The 0.17 M series was the most extensive, fewer mixtures being prepared with concentrations of 0.07 M or 0.34 M . All volumetric measurements were made with works 'class A' graduated glassware.

Ammonium, calcium, lithium, and sodium dichromates are sufficiently soluble that 0.17 M solutions could be prepared by using 11.21 g., 10.88 g., 11.30 g., and 12.66 g. respectively for 500 ml. of each solution. Owing to their low solubilities, barium, mercuric, and silver dichromates were studied as saturated aqueous solutions. Only the ammonium dichromate was of analytical grade. The others were of 'laboratory reagent' quality. For comparison with the latter, 0.17 M solutions of 'laboratory reagent' potassium dichromate and chromium trioxide were prepared.

Hydrogen-ion concentrations were determined with a Marconi type TF 717A mains-operated pH meter equipped with a saturated-potassium chloride calomel reference electrode, a glass electrode, and a temperature compensator (Marconi Instruments Ltd., St. Albans, Hertfordshire). To ensure stability, the instrument was left operating continually. It was standardized

frequently against both 0.05 M potassium hydrogen phthalate (pH 4.00) and the buffer provided for use with the pH meter (Marconi, batch 016W, pH 6.46, and batch B167, pH 6.49). All measurements were made at least in triplicate on 100 ml. portions at room temperature (17–19° C.). The suitability of the glass electrode for measuring the pH of solutions of chromic acid and its salts is well established (Neuss and Riemann, 1934; Dole, 1941).

The same instrument was used for the oxidation potential measurements with a gold electrode and the saturated-potassium chloride calomel reference electrode. The determinations were also made in triplicate, at room temperature, on 100 ml. portions. Sufficient time, usually 45 minutes or longer, was allowed for the charge on the gold electrode to attain equilibrium with the solution (Kolthoff and Furman, 1931; Elek and Boatman, 1953). The potential of the reference electrode was taken as being 0.250 volts at 20° C. (Hewitt, 1950).

For the cytological studies, tissues were taken from adult, brown or white mice of either sex. Each animal was provided with water and wholemeal bread *ad libitum* for 24 hours, then kept for 3½–4 hours without food before being killed with chloroform. Approximately 1.5 mm. cubes of liver, kidney, and pancreas, and short lengths of the first part of the jejunum were prepared.

The 0.17 M solutions were used for fixation. Four pieces of tissue, one of each kind, were placed in a 20-ml. portion of the reagent. Three such sets of tissues were prepared for each solution tested. These were left for 18–20 hours at room temperature, being shaken occasionally at first. Hydrogen-ion concentrations and oxidation potentials were determined immediately before the tissues were added and again after they were removed the next day. In some cases measurements were also made at intervals during fixation. 250-ml., rather than 20-ml., portions were used for fixation by the 0.001 M reagents.

After fixation all sets of tissues were washed for at least 4 hours in running tap water. One set was then dehydrated in 70 per cent., 90 per cent., and absolute ethanols, cleared in xylene, and embedded in paraffin. The remaining two sets were postchromed for 2 days at 37° C., one in 20 ml. of saturated aqueous potassium dichromate, the other in a fresh 20-ml. portion of the same solution as had been used for fixation. After being postchromed, the tissues were washed, dehydrated, cleared, and embedded like the first set.

For cytological studies, paraffin sections were cut at 3 μ . General observations were made on sections stained with Ehrlich's haematoxylin and eosin. Mitochondria were selectively stained by Heidenhain's or Hirschler's iron haematoxylin methods or by Metzner's acid fuchsin/picric acid technique. With the latter, differentiation was very rapid, generally requiring only 1 or 2 minutes even when a reagent containing half the usual amount of picric acid was used. Some additional observations were made on the affinity of the fixed tissues for haematein (Hirschler's) without previously mordanting with ferric alum.

A few histochemical procedures, including treatment with acetylated Sudan black B (Casselmann, 1954) or Schiff's reagent, and the periodic acid/Schiff

(McManus, 1946), nucleal (Feulgen and Rossenbeck, 1924), and coupled tetrazonium (Pearse, 1953) tests, were applied to $10\ \mu$ paraffin sections.

OBSERVATIONS

Hydrogen-ion concentrations

Three complete series of pH measurements were made on the $0.07\ M$ and $0.34\ M$ solutions and five on the $0.17\ M$ ones. Considering that these solutions are not appreciably buffered, there is remarkably good agreement

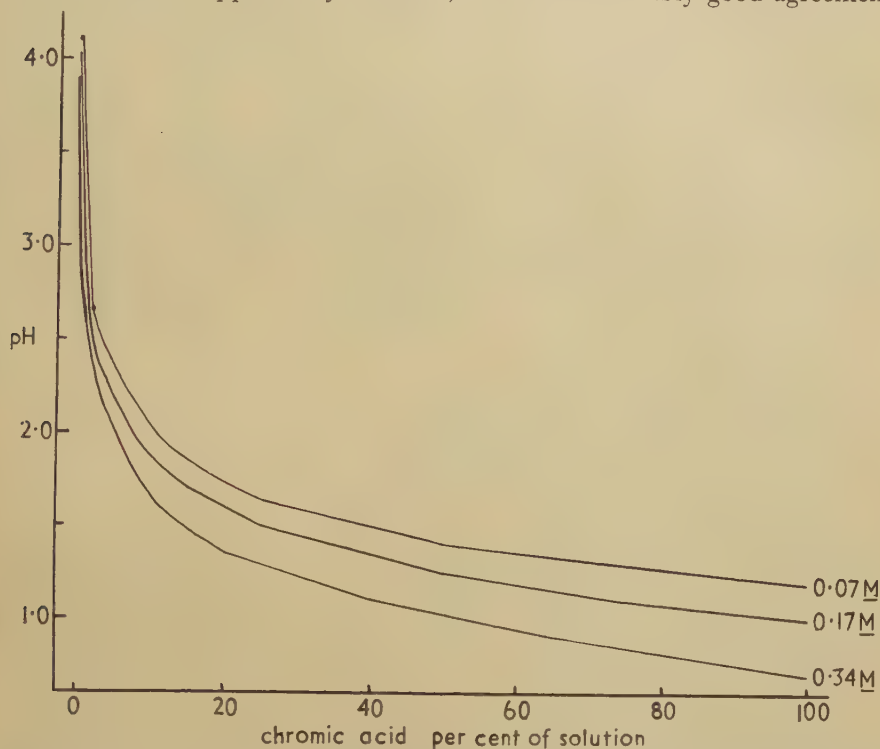


FIG. 3. Relation between pH and composition of solutions of potassium dichromate (abscissa = 0 per cent.), chromic acid (abscissa = 100 per cent.), and mixtures of the two reagents, having chromium trioxide concentrations of 0.07 , 0.17 , and $0.34\ M$. (Based on experimental observations.)

between the averages for the different series. Thus, for 2.5 per cent. potassium dichromate, none deviates more than ± 0.06 pH unit from the overall mean. For the corresponding $0.17\ M$ chromic acid, the deviation is ± 0.04 unit, and for any of the $0.17\ M$ mixtures, from -0.05 to $+0.08$ unit. The following results are recorded to the nearest 0.05 pH unit.

The mean values for $0.07\ M$, $0.17\ M$, and $0.34\ M$ potassium dichromate solutions are pH 4.10, pH 4.05, and pH 3.85, respectively. The corresponding values for the chromic acid solutions are pH 1.20, pH 0.85, and pH 0.70. These results and those for the potassium dichromate/chromic acid mixtures are presented graphically in fig. 3. A mixture of equal parts of $0.17\ M$ potassium

dichromate and 0.17 *M* potassium chromate has a pH of 6.65 while the 0.17 *M* potassium chromate alone is pH 8.80.

Two series of measurements were made on each of the other seven dichromates. The mean values for the 0.17 *M* solutions are: ammonium dichromate, pH 3.80; calcium dichromate, pH 3.45; lithium dichromate, pH 4.90; and sodium dichromate, pH 5.10. Those for the saturated solutions are: barium dichromate, pH 1.75; mercuric dichromate, pH 1.05; and silver dichromate, pH 3.00. The values for the 0.17 *M* solutions of 'laboratory reagent' grade potassium dichromate and chromic acid are pH 3.95 and pH 1.20 respectively.

A decrease in the hydrogen-ion concentration occurs during the fixation of tissues, largely in the first 3–4 hours. The change is greatest in the solutions of highest initial pH, averaging 0.8 pH unit for 2.5 per cent. potassium dichromate but only 0.1 unit for the corresponding chromic acid. For any given reagent, however, the change in pH varied considerably between trials, presumably because no attempt was made to keep the portions of tissues identical. Similar, or slightly smaller, changes in hydrogen-ion concentration occur during postchromation.

Oxidation potentials

Five series of oxidation potential measurements were made on certain of the chromic acid or potassium dichromate solutions. With the gold electrode, reproducibility of results was only moderately good (compare Hewitt, 1950). The mean values are recorded to the nearest 0.02 volt. The effect of dilution is small, usually within the limits of experimental error. Over the concentration range, 0.07–0.34 *M*, the mean potential is 1.10 volts for chromic acid and 0.76 volt for potassium dichromate. For 0.17 *M* mixtures, where 50 per cent. of the solute is chromic acid, the potential is 1.06 volts; 5 per cent., 0.96 volt, and only 0.5 per cent., 0.86 volt. No measurements were made on the seven other dichromates.

During the fixation of tissues, there is little (0.015 volt) or no significant alteration in the potentials of either 0.17 *M* chromic acid or 0.17 *M* potassium dichromate. With chromic acid, a slight decrease (0.07 volt) occurs during 2 days' postchroming at 37° C. No significant change in the potential of potassium dichromate occurs in the same time.

Cytological observations

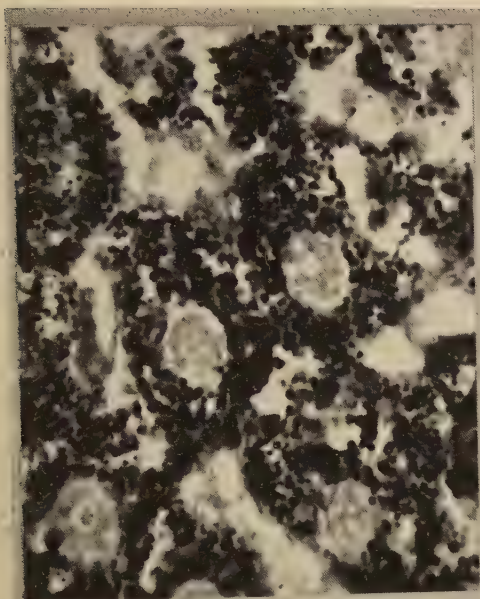
The morphological features of the various cells are not appreciably affected by postchroming the fixed tissues. Especially for the mitochondria and nuclei, the intensity of staining and the ease with which differentiation may be

FIG. 4 (plate). Photomicrographs of hepatic parenchymal cells of mice. Tissue blocks were fixed in 0.17 *M* potassium dichromate (A and B) or 0.17 *M* chromic acid (C and D) and post-chromed in saturated aqueous potassium dichromate. Paraffin sections were cut at 3 μ and stained as follows:

A, Metzner's acid fuchsin and picric acid.

B and D, Ehrlich's haematoxylin and eosin.

C, Heidenhain's iron alum and haematoxylin.

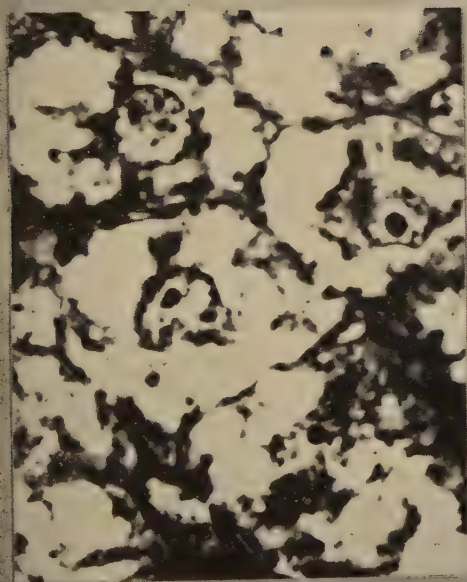


A



B

10 μ



C



D

FIG. 4
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effected are influenced by the solution used for postchromation. Generally, staining is impaired by treatment with the more acidic reagents. The staining reactions described below are for tissues postchromed in saturated aqueous potassium dichromate.

As noted by Zirkle (1928, *a* and *b*) with *Zea mays*, the cytological findings in mouse tissues fixed by the anionic chromium reagents fall into two broad groups represented by those produced by chromic acid and by potassium dichromate respectively. An intermediate group may be distinguished in some instances. The observations on the hepatic parenchymal cell will be described in detail. Generally, the findings in the other types of cells are so similar that they will be only summarized.

Chromic acid. The hepatic parenchymal cell fixed by chromic acid (fig. 4, *c* and *d*) is usually somewhat distorted in outline. Its cytoplasm is precipitated about the periphery of the cell and around its nucleus. The intervening space is occupied by some granules or bridged by strands or bands of precipitated cytoplasm. Nowhere are the mitochondria evident. The nucleus is filled with coarsely granular or reticular material. The nucleolus is usually well preserved.

All structures are stained by only the acid fuchsin in Metzner's method or the eosin in Ehrlich's haematoxylin/eosin preparations. By either of these methods, the nuclear and cell-membranes are poorly demonstrated. The cytoplasm stains moderately well, the nucleus only weakly. The nucleolus, however, is intensely coloured by acid fuchsin. With the iron haematoxylin methods, the cell and nuclear membranes are clearly defined but the cytoplasm is quite irregularly stained, the nucleus not at all. The nucleolus is moderately evident. Unmordanted haematein stains the cytoplasm weakly and irregularly, the nucleus slightly more intensely.

In the cells of the pancreatic acinus and the intestinal epithelium fixed by chromic acid, distortion of cellular outline is negligible. The individual cells in the proximal convoluted tubules of the kidney are difficult to distinguish. In all three cells, the cytoplasm and nuclear contents are usually more granular or finely reticular than in the hepatic parenchymal cell similarly fixed. The zymogen granules of the pancreatic cell are stained reddish-yellow by Metzner's method. They are just demonstrable with eosin but not by the iron haematoxylin methods. The cell membrane of the intestinal cell is well defined. In none of the cells can the mitochondria be seen. In the renal cell, the nucleus contains relatively less demonstrable material than in the other cells. The nucleolus, however, is present and quite well stained by acid fuchsin.

Potassium dichromate. The components of the hepatic parenchymal cell are well preserved and differentially stained after fixation by potassium dichromate (fig. 4, *A* and *B*). The cell outline is regular. The mitochondria vary in size. Most of them are spherical. The cytoplasm and nuclear contents are homogeneous, or very finely granular, with occasional small holes. The nucleolus is small and usually surrounded by a narrow, unstained 'halo'.

In sections stained by Metzner's method, neither the cell membrane nor the

nuclear membrane is evident. The cytoplasm is faintly coloured by the picric acid, the nuclei rather more intensely. The mitochondria are quite uniformly and darkly stained by the acid fuchsin. The nucleolus is not differentially stained. With the iron haematoxylin methods, the mitochondria vary in colour from bluish-grey to black. The other structures in the cell are practically unstained. With Ehrlich's haematoxylin and eosin, the nucleolus is dark purple, the nucleus lighter purple, while the remainder of the cell is pink. The cytoplasm is weakly stained, the nucleus not at all, by unmordanted haematein.

In the intestinal epithelial cell fixed by potassium dichromate, the striated border, the long, thread-like apical mitochondria and the shorter, more densely packed basal ones are well preserved. Usually, the nuclear contents are retracted from the surrounding membrane. The nucleolus is not discernible in the intestinal or renal cells but can be seen in the pancreatic acinar cell. In the latter, the zymogen granules are very faintly stained, if at all, by Metzner's method and not at all by the iron haematoxylin techniques. The mitochondria are clearly evident in these cells as well as in those of the renal tubules.

Chromic acid / potassium dichromate mixtures. With the 0.17 *M* series of potassium dichromate / chromic acid mixtures, the cytological findings depend upon the composition of the fixing solution. Where the chromic acid constitutes only a very small proportion of the total solute, not over 1 per cent., all but the most peripheral cells in a section present the typical potassium dichromate picture. Some of the cells around the edge of the section show an intermediate type of fixation. In these, the cytoplasm and nuclear contents are precipitated in a granular or reticular form, approaching that of chromic acid fixation. The mitochondria are present and the nucleus is well stained as with potassium dichromate fixation. Nuclear chromatin continues to be stained by Ehrlich's haematoxylin as long as not over 5 per cent. of the solute is chromic acid.

As the chromic acid is increased to about 10 per cent. of the total solute, the zone of intermediate fixation widens and moves inwards. It is surrounded by a rapidly broadening zone of typical chromic acid fixation. A decreasing central portion of the section retains the potassium dichromate picture. The widths of the intermediate and acidic zones are approximately proportional to the amount of chromic acid in the fixing mixture. With blocks of tissue of the size used in these studies, almost none of the dichromate picture persists when 15 per cent. of the solute is chromic acid. When it is 20 per cent., nearly the entire section presents the acid picture. The hepatic parenchymal cells around some central veins, however, are more resistant to chromic acid and, occasionally, present an intermediate picture even when 50 per cent. of the solute is chromic acid. They are resistant also when a mixture with this proportion of chromic acid is brought to the tissue by perfusion.

Fixation by the potassium dichromate / potassium chromate mixture, pH 6.65, resembles that by potassium dichromate alone. The alkaline potassium chromate by itself is unsuitable for cytological fixation.

0.001 M solutions. Tissues fixed and postchromed in 0.001 *M* solutions of chromic acid or potassium dichromate in saline are remarkably like those fixed in the more concentrated aqueous solutions. They differ appreciably from control tissues treated with 0.85 per cent. sodium chloride alone. In the latter, few cytological details are preserved.

The hepatic parenchymal cell is regular in outline after treatment with 0.001 *M* chromic acid. Rarely can the cell membrane be seen. The cytoplasm is usually moderately granular and only weakly stained. The mitochondria are not present. The thin but clearly defined nuclear membrane surrounds an almost empty nucleus. Apart from the intensely stained nucleolus, it contains only a few granules of material stained with picric acid in Metzner's method.

After fixation in the very dilute potassium dichromate reagent, the general features of the cell and its cytoplasm are the same as those just described. The mitochondria, however, are present and especially well stained by the iron haematoxylin methods. There is a larger quantity of granular material in the nucleus.

Ammonium, lithium, and sodium dichromates. Fixation of the parenchymal cell of the liver by ammonium, lithium, or sodium dichromate closely resembles that by potassium dichromate. The cell and nuclear membranes are practically imperceptible. The cytoplasm is homogeneous or finely granular. The mitochondria are round but vary in size, especially after fixation in sodium dichromate. They are intensely and, usually, uniformly stained by acid fuchsin or by the iron haematoxylin methods. The nucleus is well filled with finely granular material which is often slightly retracted from the membrane after fixing in sodium dichromate. After fixation by lithium dichromate, the nucleolus appears optically more dense than the other contents of the nucleus. It is surrounded by a moderately wide, clear zone or 'halo'. The nucleolus is just differentially stained by acid fuchsin, better by iron haematoxylin. After fixation with sodium dichromate, the nucleolus is more difficult to see, and after ammonium dichromate, it is only just distinguishable.

A 0.17 *M* potassium dichromate/potassium chromate mixture, prepared to have a hydrogen-ion concentration (pH 5.0) intermediate between that of lithium dichromate (pH 4.90) and that of sodium dichromate (pH 5.10), gives fixation practically indistinguishable from that by either of these dichromates.

Barium, calcium, mercuric, and silver dichromates. Fixation by the dichromates whose solutions are more acidic (namely, barium, calcium, mercuric, and silver dichromates) generally resembles that by chromic acid. The cell is remarkably large after fixation in saturated aqueous barium dichromate. The cell and nuclear membranes are just discernible. The cytoplasm is coarsely granular and contains many small holes with darkly staining rims suggestive in size and distribution of distended, vacuolated mitochondria. The nuclear contents are coarsely granular. The nucleolus stains more intensely with acid fuchsin and is usually irregular in outline. Nothing is stained by Ehrlich's haematoxylin.

After fixation in 0.17 *M* calcium dichromate, the cell is rather irregular in outline. About its periphery, the finely reticular cytoplasm contains moderately large holes which are not present near the nucleus. The chromatin and the nucleolus are reddish with Ehrlich's haematoxylin and eosin. The remainder of the findings in the various types of cells, except those of the renal tubules, are the same as after fixation with chromic acid. In the cells of the proximal convoluted tubules of the kidney, however, the picture is dominated by large structures resembling mitochondria. These are intensely stained by acid fuchsin and by the iron haematoxylin methods.

Cellular distortion is less after fixation in saturated aqueous mercuric dichromate than after chromic acid. The cell and nuclear membranes are more easily seen. The cytoplasm tends to be more granular. Otherwise, the findings are similar.

When fixed in saturated aqueous silver dichromate, the cell varies from being somewhat shrunken and irregular in outline to being appreciably swollen and regular in outline. The cytoplasm is usually a fine, open reticulum with a few, more darkly staining specks. Unstained, the homogeneous nuclear contents are brownish. They are quite intensely coloured by Ehrlich's haematoxylin. The nucleolus is smaller than usual but stains well with the alum haematoxylin or with acid fuchsin.

Histochemical observations

Lipids are demonstrable by acetylated Sudan black B in the adipose tissue around the pancreas and the renal pelvis, and in some intestinal lacteals, whether chromic acid or potassium dichromate was used as fixative.

Schiff's reagent alone gives a faint, diffusely positive reaction with tissues fixed in chromic acid, in mixtures of which at least 5 per cent. of the solute is this acid, and in some of the more acidic dichromates. In some instances, such structures as the striate border of the intestinal epithelial cell, the brush border of the renal tubule, the renal basement membrane, and the cytoplasm of the hepatic parenchymal cell react slightly more intensely with the Schiff's reagent. The individual glycogen granules in the liver cell, however, are not discernible. The nuclei of most cells react with the reagent to a variable degree, especially after fixation in chromic acid. In tissues which have been postchromed in the fixing reagent, these reactions are usually less intense than in similarly fixed tissues postchromed in saturated aqueous potassium dichromate. Sometimes, the structures no longer give a positive reaction.

The nucleal reaction is most intensely positive in the nuclei of the cells fixed in the more acidic reagents. Even here, however, it is nearly indistinguishable in intensity from the unhydrolysed controls.

There is little difference between sections treated with periodic acid and their unoxidized controls. Hepatic glycogen is poorly preserved by any of the fixing reagents. As with Schiff's reagent alone, diffuse reactions of moderate intensity are obtained after potassium dichromate. With the more acid solutions, the reaction becomes weaker and more patchy. After postchroming

in these reagents, little or no positive reaction is obtained. Structures such as the striate border of the intestinal epithelial cell, the brush border of the cells of the renal tubules, and the basement membrane of the kidney sometimes give more intense reactions than in the control sections but usually not after the more acidic fixing reagents.

With the coupled tetrazonium test, the tissues are coloured from a pale, brownish-yellow after chromic acid, to a slightly more intense yellowish-brown after potassium dichromate. In no instance are the reactions the typical reddish-brown expected with the coupled tetrazonium test.

DISCUSSION

The values reported here for the hydrogen-ion concentrations of the various solutions of chromic acid and its salts agree well with those published by previous investigators. Lassek and Lunetta (1950a), using the glass electrode for their measurements, also reported pH 1.20 for 1 per cent. chromic acid. From colorimetric estimations, Zirkle (1928a) concluded that the pH of 2.5 per cent. chromic acid is less than 1.0. This agrees with pH 0.70 for 3.4 per cent. and pH 0.85 for 1.7 per cent. solutions of the acid as determined with the glass electrode.

When freshly prepared, 3 per cent. potassium dichromate was found to have a pH of 3.9 by Lassek and Lunetta (1950a). After one month, the same solution had a pH of 4.2. From this, they concluded that the solution is unstable. During the present studies, the pH of 2.5 per cent. potassium dichromate solutions did not vary appreciably from pH 4.05 even after storage for 4 months in a 'Pyrex' flask. It is probable that the change observed by Lassek and Lunetta was due to the leaching of alkali from the glass container. Solutions of potassium dichromate are so stable that they can be used as oxidimetric standards. Zirkle's estimate for 2.5 per cent. potassium dichromate was pH 4.4.

Although Zirkle's other solutions of dichromates differed in concentration and, sometimes, in mode of preparation from those used in this investigation, most of his estimates of their hydrogen-ion concentrations are nearly the same as those reported here. In the cases of calcium and sodium dichromates, however, the differences are appreciable. His values of pH 5.6 and pH 4.4 respectively are in contrast to the pH 3.45 and pH 5.10 of the solutions prepared for this study. Such differences, however, probably reflect differences in the relative purities of the salts. Zirkle did not prepare a solution of silver dichromate alone.

The influence of the purity of the reagents upon the hydrogen-ion concentrations of their solutions is further illustrated by the data for the 0.17 *M* 'analytical' and 'laboratory reagent' grade potassium dichromate and chromic acid. The solution of the less pure dichromate is rather more acidic, pH 3.95 as compared to pH 4.05. The less pure acid has a higher pH, 1.20 instead of 0.85. In neither case is the difference sufficient to alter the type of cytological fixation.

The results of the oxidation potential measurements show that chromic acid is a stronger oxidizing agent than potassium dichromate. The value of 1.10 volts for chromic acid is comparable with those reported by Luther (1899), Kolthoff (1919), and Kolthoff and Furman (1931). The influence of hydrogen-ion concentration is evident in the lower potential (0.76 volt) for potassium dichromate. The 0.34 volt difference is rather greater than the changes noted by Luther or Kolthoff for a similar decrease in hydrogen-ion concentration. These authors, however, added other acids or salts to effect the changes in pH.

Even with fivefold dilution, only a small change in potential is to be expected. In the case of potassium dichromate, the pH increases by only 0.25 unit with such dilution and the ratio of the dichromate and chromic ions remains unchanged. Consequently, the theoretical potential change would be only 0.03 volt. On the basis of Luther's and Kolthoff's observations, the actual change would be only about 0.01 volt. Such was the magnitude of the changes with dilution observed during these investigations.

The observations on the changes in hydrogen-ion concentrations and oxidation potentials which occur during fixing and postchroming of tissues indicate something of the fundamental processes involved. During fixation, there is an early, slight neutralization of some of the acidity of the reagent by the tissues. Although oxidation of certain tissue constituents is occurring, as shown by some of the histochemical observations, this is not great enough to effect any appreciable change in the oxidation potential. This lack of change can be attributed to the large excess of fixing solution used in these experiments.

During postchroming there is some neutralization by the fixed tissues. Oxidation proceeds more rapidly and to a greater extent at the higher temperature. Consequently, within the 48-hour period, there is a decrease in the oxidation potential of the more active oxidizing agent, chromic acid. If the time is extended, a comparable decrease in potential also occurs with potassium dichromate. As a result of these oxidative processes, increasing amounts of chromic ion are produced. Thus, both anionic and cationic chromium become available for reaction with tissue constituents during fixing and postchroming with chromic acid or dichromate solutions.

The cytological studies reported here, like those by Zirkle (1928, *a* and *b*), have concerned the morphology of cells and their components after being subjected not only to fixation and postchromation but also to dehydration, paraffin embedding, and staining. No attempt has been made to correlate the final appearances with the structure of living cells. For this, the sequence of changes induced by chromic acid or its salts would have to be studied by starting with living cells.

In general, the morphological characteristics of fixation by chromic acid and its salts are similar whether studied in the root tips of *Zea mays* (Zirkle, 1928, *a* and *b*) or in various mammalian tissues. After chromic acid, the cytoplasm appears disorganized and the mitochondria are absent. After

potassium dichromate, however, the cytoplasm is quite homogeneous and the mitochondria are well preserved. Both reagents fix the nucleolus but its staining properties as well as those of the rest of the nuclear contents vary with the fixing solution. Barium, calcium, mercuric, or silver dichromate acts in much the same way as does chromic acid. Fixation by ammonium, lithium, or sodium dichromate resembles that by potassium dichromate. The intermediate type of fixation observed with certain potassium dichromate / chromic acid mixtures was not described by Zirkle (1928, *a* and *b*) possibly because the root tips which he used were much smaller in cross-section than the blocks of liver and other tissues used in this investigation. The occurrence of a zone of such fixation might be related to partial neutralization of the acidic fixing reagent as it diffuses into the block of tissue.

There are two prominent features of fixation by anionic chromium reagents whether this is studied in plant or animal tissues. The first is the correlation between the type of fixation and the properties of the fixing solutions, notably their hydrogen-ion concentrations. Those reagents giving the potassium dichromate type of fixation have a pH of 3.8 or greater, those giving only the chromic acid type, pH 3.4 or less. At least some features of the intermediate type of fixation can be observed with potassium dichromate / chromic acid mixtures around pH 3.6. Zirkle (1928*a*) similarly observed that the transition between potassium dichromate and chromic acid types of fixation occurs at approximately the hydrogen-ion concentration of the potassium dichromate reagent, which he estimated colorimetrically to be pH 4.4.

The second prominent feature is that the transition between the two types of fixation occurs in or slightly above the range of hydrogen-ion concentrations where the isoelectric points of many tissue proteins occur. This is in keeping with the known properties of proteins. Around their isoelectric points, their solubilities and their reactivities with other anions and cations undergo pronounced changes. The fact that the transition point is somewhat more acidic than the isoelectric point of some of the proteins might be related to the partial neutralization of the fixing agent as it enters the cell so that the reaction at the site of fixation within the cell will be slightly less acidic than that of the solution in which the tissue is immersed.

The morphological differences between protein mixtures fixed at hydrogen-ion concentrations on either side of their isoelectric points is well illustrated by the observations on cytoplasm described above and also by Zirkle (1928, *a* and *b*). A comparable difference in the appearance of liver and brain homogenates precipitated by chromic acid and by potassium dichromate was noted by Lassek and Lunetta (1950*b*). The conversion of apparently homogeneous protein solutions into heterogeneous networks by fixation was first clearly demonstrated by Hardy (1898).

Mitochondria are fixed by only the less acidic dichromates of ammonium (pH 3.80), lithium (pH 4.90), potassium (pH 4.05), or sodium (pH 5.10). With mixtures of potassium dichromate and chromic acid, the mitochondria in at least the peripheral cells of a block of liver are destroyed when the pH

is lowered to even 3.60, while fixation by calcium dichromate (pH 3.45) is of the chromic acid type except for the cells of the renal proximal convoluted tubules. Such disintegration of mitochondria might be attributed to the denaturation of their constituent proteins and the solubilization of the pentose nucleic acids and nucleotides with a release of the phospholipids. These changes can occur under various conditions including higher hydrogen-ion concentrations (Claude, 1949). The possibility that the disintegration of the mitochondria is not solely due to the increased acidity is suggested by their resistance to 0.1 *N* hydrochloric acid (pH 1.0) (Casselman and Jordan, 1954). The importance of the anion should not be overlooked. For example, mitochondria do not survive the effects of acetate ions over a wide range of hydrogen-ion concentrations (Zirkle, 1928*b*; Casselman, unpublished).

The effects of chromic acid and certain dichromates upon nuclei was studied by Flemming (1880), who concluded that only chromic acid should be used for fixation when the nuclei are to be studied. All dichromates, he believed, are detrimental. The problem was investigated in more detail by Burchardt (1897). He found that certain dichromates, including those of potassium, sodium, lithium, and ammonium, destroyed the nuclei but preserved the surrounding cytoplasm well. Others, such as barium and calcium dichromates, preserved the nuclei but destroyed the cytoplasm. The observations reported by Zirkle (1928, *a* and *b*) and those reported here are in agreement with Burchardt's findings.

Burchardt (1897) also studied fixation by very dilute solutions and found that with 1:1,500 or more dilute chromic acid, the nuclei become granular, whereas they are irregularly lumpy after 1:1,000 calcium dichromate and quite homogeneous after 1:1,000 potassium dichromate. The effects of the more dilute 0.001 *M* chromic acid and 0.001 *M* potassium dichromate were similar but less marked.

In the various mouse tissues, the nucleolus is fixed by each of the reagents studied regardless of the hydrogen-ion concentration of the solution. The staining properties of the nucleolus, however, depend upon the fixing agent used. This structure has an affinity for acid fuchsin and for the iron haematoxylin after chromic acid and the more acidic dichromates (barium, calcium, mercuric, and silver dichromates). Although the nucleolus is still present after fixation in the less acidic reagents (ammonium, lithium, potassium, and sodium dichromates), it does not retain acid fuchsin or iron haematoxylin during differentiation, but it can be progressively stained with Ehrlich's haematoxylin. In the mouse tissues, no instance was observed of the extreme hardness of the nucleoli that had been noted by Zirkle (1928*a*) in plant tissues. Fixation by lithium dichromate did not produce the nucleolar 'pseudopodia' which he observed.

The tests with Schiff's reagent clearly demonstrate the hydrolytic and oxidative properties of chromic acid. Nuclear deoxypentose nucleic acid is hydrolysed as in the nucleal test so that the nuclei give a positive reaction which is not appreciably intensified by first treating the sections with hydro-

chloric acid. Various carbohydrates initially undergo oxidative alpha-glycol cleavage to yield aldehydes—the reaction being the same as in Bauer's test (Bauer, 1933). Unlike periodic acid, however, chromic acid can further oxidize the aldehydes to products which do not react with Schiff's reagent. This is illustrated by the weak or negative reactions in tissues postchromed in the more acidic and, therefore, more strongly oxidizing reagents. The results with the periodic acid / Schiff tests were practically the same as in the untreated controls because the fixing reagent had already acted on the compounds which might have undergone oxidative cleavage by periodic acid.

That some of the tissue lipids resisted paraffin embedding is additional evidence of the oxidizing properties of chromic acid and dichromates. They oxidize unsaturated lipids to substances which are insoluble in the usual fat solvents. This is the basis of Ciaccio's (1909) method for preserving lipids for demonstration in paraffin sections. Similarly, pure unsaturated fatty acids can be converted to substances resembling ceroid pigment (Casselman, 1951). The variation in the oxidation potential of chromic acid and dichromates with change in concentration of hydrogen ions has been utilized by Elftman (1954) in his technique of 'controlled chromation'. Lipid-bound chromic ions are probably responsible for the reaction of postchromed lipids with dyes in staining techniques such as that devised by Weigert (1884) for myelin and in the acid haematein test for phospholipids (Baker, 1946).

The coupled tetrazonium test depends upon the tyrosine, tryptophane, and histidine in tissue proteins. These substances are, to some degree, susceptible to oxidation. During fixation and postchroming by the solutions used in these experiments, it appears that these amino acids were so much altered that little remained to react with the diazotized benzidine reagent of the test.

These histochemical observations are in accord with the known oxidative and hydrolytic properties of chromic acid and dichromates and with the known responses of certain protoplasmic constituents to these reagents. In so far as the morphological features of cells fixed by these compounds are concerned, however, probably more important are ionic interactions and complex formations, and the precipitation reactions sometimes associated with these processes. The exact chemical mechanism of cytological fixation by chromic acid or its salts awaits elucidation. Its analogy with chrome tanning has been suggested by some authors. The fixation procedure does resemble the first part of 'two-bath' chrome tanning (McLaughlin and Theis, 1945), but whereas in tanning, the chromic cation plays the major role, in cytological fixation, chromium anions are present in almost infinitely greater proportions and, undoubtedly, are much more important. The interactions between various proteins and certain small anions have been studied quite extensively (Klotz, 1953) although not with specific reference to chromium. The small amount of chromic ion formed by reduction during fixation can also react with protoplasmic constituents such as proteins and nucleic acids.

The observations of Hermann and Speck (1954) show that under such conditions the chromic ion can also form remarkably stable complexes with nucleic acids or nucleo-proteins.

I am most grateful to Dr. John R. Baker for his advice and ever inspiring guidance during these studies. It is a pleasure to acknowledge the technical assistance of Miss Barbara M. Jordan.

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The Relative Oxidizing Properties of Certain Reagents and Mixtures used for the Fixation of Tissues

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SUMMARY

The oxidation potentials of the fixing reagents studied range from 1.10 V for chromic acid to 0.23 V for formaldehyde. Potassium dichromate, mercuric chloride, and osmium tetroxide have intermediate values. Commonly used mixtures prepared from these compounds also exhibit a range of potentials from 1.00 V for Champy's to 0.15 V for Regaud's. The oxidation potentials of Altmann's, Champy's, Flemming's, Helly's, and Zenker's fluids remain relatively constant for at least 1 day, whereas those of Regaud's and Sanfelice's fluids undergo appreciable change.

DURING an investigation of cytological fixation by reagents containing anionic chromium, the oxidation potentials of various solutions of chromic acid and potassium dichromate were determined (Casselmann, 1955). Because no previous reports regarding the redox potentials of fixing solutions could be found in the literature, these studies were extended to include certain other reagents and mixtures. The results are presented here.

METHODS AND MATERIALS

With the exception of osmium tetroxide, analytical grade reagents were used. All solutions were prepared in distilled water. Measurements were made on formalin which had been 'neutralized' with calcium carbonate as well as on the 'unneutralized' solution. Only the former was used in the preparation of mixtures.

Both hydrogen-ion concentrations and oxidation potentials were measured. These were determined with a Marconi type TF 717A mains-operated pH meter (Marconi Instruments Ltd., St. Albans, Hertfordshire). To ensure stability, the instrument was left operating continually. For the pH measurements, it was equipped with a saturated-potassium chloride calomel reference electrode, a glass electrode, and a temperature compensator. It was standardized frequently against the buffer provided for use with the pH meter (Marconi, batch 016W, pH 6.46) and occasionally against 0.05 M potassium hydrogen phthalate (pH 4.00). For the oxidation potential measurements, the same calomel electrode and a gold electrode were used. All measurements were made at least in duplicate at room temperature (17°–19° C.) and, usually, on 50-ml. portions of the solutions or mixtures. In the case of the oxidation-potential measurements, sufficient time (45–75 minutes) was allowed for the

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charge on the gold electrode to attain equilibrium with the solution (Kolthoff and Furman, 1931; Elek and Boatman, 1953). The potential of the reference electrode was taken as being 0.25 V at 20° C.

Measurements of hydrogen-ion concentrations and oxidation potentials were made on solutions of the primary reagents as they are used in fixing mixtures: 1 to 3.4 per cent. chromic acid, 10 per cent. formalin (approximately 4 per cent. formaldehyde), 1 to 5 per cent. mercuric chloride, 2 per cent. osmium tetroxide, and 2.5 to 5 per cent. potassium dichromate.

Similar measurements were made after the addition of each solute during the preparation of the fixing mixtures and, finally, after each mixture had been left for 1 day at room temperature. The following mixtures were studied: Altmann's, Champy's, Flemming's, Helly's, Regaud's, Sanfelice's, and Zenker's.

OBSERVATIONS

The observed hydrogen-ion concentrations and oxidation potentials of solutions of the primary fixing reagents are presented in table 1, which also

TABLE I
Primary fixing reagents

Reagent	pH	E	Reaction	E°
	%	V		V
chromic acid, 3.4	0.80	1.10	$\text{Cr}_2\text{O}_7^{--} + 14\text{H}^+ + 6e^- \rightleftharpoons 2\text{Cr}^{+++} + 7\text{H}_2\text{O}$	1.33
1.7	1.05			
1.0	1.20			
potassium dichromate, 5.0	3.70	0.78		
3.0	3.85			
2.5	3.85			
mercuric chloride, 5.0	3.75	0.75	$2\text{Hg}^{++} + 2e^- \rightleftharpoons \text{Hg}_2^{++}$	0.92
1.0	3.25			
osmium tetroxide, 2.0	(4.75)	0.64	$\text{OsO}_4 + 8\text{H}^+ + 8e^- \rightleftharpoons \text{Os} + 4\text{H}_2\text{O}$	0.85
formaldehyde, 3.6 (10% formalin):		0.23	$\begin{cases} \text{HCOOH} + 2\text{H}^+ + 2e^- \rightleftharpoons \text{HCHO} + \text{H}_2\text{O} \\ \text{HCHO} + 2\text{H}^+ + 2e^- \rightleftharpoons \text{CH}_3\text{OH} \end{cases}$	0.056 0.19
unneutralized	3.40			
neutralized	4.55			

includes the reactions undergone by each reagent and its standard oxidation potential (Latimer, 1952). The pH of the osmium tetroxide solution depends upon that of the water in which it is dissolved.

The hydrogen-ion concentrations and potentials recorded at each step in the preparation of Zenker's, Helly's, and Champy's mixtures are summarized in table 2. Table 3 presents the observations on the various mixtures when they were freshly prepared and, again, 1 day later. During the first 3-4 hours

TABLE 2

Stages in the preparation of Zenker's, Helly's, and Champy's fluids

	pH	E
		V
100 ml. 2.5% potassium dichromate	3.85	0.78
+ 1 gm. sodium sulphate	4.00	..
+ 10 gm. mercuric chloride	3.60	0.76
57 ml. above 'stock' mixture	3.60	0.76
+ 3 ml. glacial acetic acid (Zenker's)	2.50	0.83
57 ml. 'stock' mixture	3.60	0.76
+ 3 ml. neutralized formalin (Helly's)	3.70	0.74
35 ml. 3% potassium dichromate	3.85	0.78
+ 35 ml. 1% chromic acid	1.80	1.03
+ 20 ml. 2% osmium tetroxide (Champy's)	1.80	1.00
20 ml. 2% osmium tetroxide	(4.75)	0.64
+ 35 ml. 3% potassium dichromate	3.90	0.76
+ 35 ml. 1% chromic acid (Champy's)	1.80	1.00

TABLE 3

Fixing mixtures

Mixture	pH		E	
	When prepared	After 1 day	When prepared	After 1 day
Champy's (see table 2)	1.80	1.80	V	V
Flemming's	1.45	1.30	1.00	1.00
15 vol. 1% chromic acid				
4 vol. 2% osmium tetroxide				
1 vol. glacial acetic acid				
Zenker's (see table 2)	2.50	2.50	0.83	0.83
Helly's (see table 2)	3.70	4.20	0.75	0.70
Altmann's	4.00	4.00	0.70	0.70
1 vol. 5% potassium dichromate				
1 vol. 2% osmium tetroxide				
Sanfelice's	..	3.00	(0.63)	0.55
16 vol. 1% chromic acid				
1 vol. glacial acetic acid				
8 vol. formalin				
Regaud's	4.70	4.85	(0.19)	0.15
8 vol. 3% potassium dichromate				
2 vol. formalin				

after preparation, there is a steady decline in the potentials of the mixtures containing formaldehyde, especially Regaud's and Sanfelice's fluids. The values within the parentheses are those at the end of the first hour. A moderate

colour change from orange to brown was noted for Regaud's fluid and a much more pronounced one, from orange to dark greenish-brown, for Sanfelice's.

DISCUSSION

These observations emphasize the range of oxidative properties to be found among some of the more commonly used fixing reagents and mixtures, ranging from the quite strongly oxidizing chromic acid and Champy's and Flemming's fluids to the more reductive formalin and Regaud's fluids. The great changes in potential occurring in mixtures such as Sanfelice's and Regaud's, like the changes in the colours of these solutions, indicate appreciable alterations in the composition of the mixtures. The changes will be largely oxidation of formaldehyde, initially to formic acid, with reduction of the chromic acid or dichromate to the trivalent chromium cation. In these mixtures, therefore, fixation might not be entirely by anionic chromium.

The role of oxidative or reductive processes in general cytological fixation is still unknown, especially with regard to any morphological changes which are induced. Certain specific instances where these processes are important can be cited; for example Ciaccio's method for rendering lipids less soluble in organic solvents and other applications of 'postchroming' depend upon oxidation. Oxidative cleavage of certain tissue carbohydrates, which may be histochemically undesirable, can occur with certain mixtures containing chromic acid, in the same way as it occurs under more controlled conditions in Bauer's test.

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Staining Differences in Cell Nuclei

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With one plate (fig. 1)

SUMMARY

If the parenchymal cells of the liver of the rat, or certain other cells, are stained with a modified Mallory stain or with iron haematoxylin, the nuclei are stained in two different ways. This effect may be observed after various fixatives.

Direct microscopical observation shows that when part of a nucleus is cut away by the microtome-knife, the part of it remaining in the section stains differently from an intact nucleus. The probability of a nucleus being wholly or only partly contained in a section of a particular thickness can be calculated.

Counts made in sections of various thicknesses show that the proportion of nuclei stained in the different ways is consistent with the calculated probability.

It is suggested that the nuclear membrane in the fixed state presents a relatively low permeability.

SHEININ and Davenport (1931) observed in liver cells two types of nuclei that may be distinguished by differential staining. This result was obtained after fixation of the tissue in a complex mixture containing potassium dichromate, sodium sulphate, mercuric sulphate, sulphuric acid, and acetic acid, and staining with Mallory's triple connective tissue stain. Although no other difference could be found between the cells with nuclei differentially stained, Sheinin and Davenport postulated the existence of varying physiological states of the cells.

Recently, Parr, Mossberg, Rosenzweig, Breslar, and Clark (1953), using a technique derived from Mallory's stain, were able to confirm these results. Furthermore, they observed that in rats starved or on protein-free diet, there is a shift in the relative proportion of the two types of nuclei. They suggested unknown histochemical differences between the two types of nuclei; however, no definitive explanation was given for the modification caused by the changes in diet.

Long ago, I chanced to observe striking differences between nuclei in tissues stained with Heidenhain's iron haematoxylin. Some nuclei, generally few in number, strongly resist the destaining action of the iron alum and remain heavily stained, while others are nearly destained. Although no thorough search in the literature was made, it appeared that such 'dark' nuclei in iron haematoxylin preparations have occasionally been observed by numerous authors. An attempt to trace an explanation for this peculiar phenomenon was made. However, no positive evidence concerning the

mechanism of the production of 'dark' nuclei could be found, and for this reason the investigation was discontinued. It appeared to the author that the 'light' nuclei described by Parr, Mossberg, Rosenzweig, Breslar, and Clark in modified Mallory preparations might correspond to the 'dark' nuclei observed in iron haematoxylin preparations. The observations of Parr and others seemed further to indicate the possibility of increasing experimentally the proportion of these nuclei. In the present paper an attempt is made to find an explanation for the appearance of differently stained nuclei in tissues.

MATERIAL AND METHODS

The animals used in these experiments were young adult male rats, weighing about 250 g. and fed on a standard complete diet.

For the study of the action of various fixatives on the staining properties of the nuclei, fragments of the same liver were fixed for 24 hours in the following fluids: Bouin; Zenker; Susa; Helly; Carnoy; alcohol formalin, 90:10; alcohol formalin acetic acid, 85:10:5; 10 per cent. formalin in a 0.9 per cent. solution of NaCl buffered with M/50 phosphate buffers at pH 3.5 and pH 7.0. All the pieces of tissue were dehydrated and embedded in paraffin in an automatic inclusion apparatus. Sections were cut at 8 μ , 10 μ , or 12 μ . In order to eliminate differences due to uneven handling, the sections from the nine fragments treated with the different fixatives were mounted on the same slide.

Two staining methods were used as a routine to differentiate the two types of nuclei. The first was Mallory's stain as modified by Parr, Mossberg, Rosenzweig, Breslar, and Clark. A variety of modifications of Mallory and Azan stains was tried, but none proved to be superior to the procedure recommended by Parr and his associates, and the latter was adopted. The second was Heidenhain's iron haematoxylin, used as follows: mordanting in 4 per cent. iron alum for 24 hours at laboratory temperature (26° C.); staining in well-ripened Regaud's haematoxylin; differentiation in 2 per cent. iron alum. For the special purpose of demonstrating the two types of nuclear staining, the time used for the differentiation is not critical. As was mentioned before, the 'dark' nuclei resist destaining so energetically that they appear intensely stained even when the other ones are nearly completely destained. The contrast obtained with the latter method is better than with the Mallory technique.

For studying the influence of the thickness of the section, sections of the same block of liver fixed in alcohol formalin acetic acid were cut at 4, 6, 8, 10, 12, 14, and 16 μ and mounted together on the same slide. This was done in order to make the staining as uniform as possible.

Counts of the nuclei were made in the following way: A reticule dividing the field into twelve parts was placed in the ocular of the microscope. Since, according to Parr and others, nests of 'light' nuclei were more

numerous near the central veins, precautions were taken to ensure a random sampling in the liver. Keeping this in view, the nuclei were counted in successive fields, always in the same direction, beginning at one surface of the liver and continuing vertically to the other surface. Usually 600 to 750 nuclei were counted. The counts were reduced to percentages of the two types of nuclei. Upper and lower limits of the confidence-interval for a probability of 95 per cent. were calculated from the tables of the confidence-intervals given by Snedecor (1946) for the binomial distribution, by interpolating the values of the table for the number of items in the count. The significance of differences between the proportion of both types has been tested by the χ^2 test based on the null hypothesis, using the tables of distribution of the probability of χ^2 established by Fisher (1950).

RESULTS

Identity of the 'yellow' nuclei in modified Mallory preparations and the 'dark' nuclei in iron haematoxylin preparations

A first series of observations was made to ascertain whether the nuclei differentially stained by the methods of Sheinin and Davenport and of Parr and others are the same that are differentiated by Heidenhain's iron haematoxylin.

In the preparations treated with the method of Parr and others, most of the nuclei are stained blue, while others are golden yellow. The inspection of the preparations gave the impression that these 'yellow' nuclei may be identical with the nuclei that remain heavily stained in black in strongly destained iron haematoxylin preparations.

However, since no method was found that would make it possible to stain the same cells by the two staining methods, one after the other, some doubt remained as to the identity of the nuclei selectively stained. In order to give a valid proof of this identity, a statistical analysis of the results of comparative counts was made.

It will be shown later that it is possible to modify at will the ratios of both types of nuclei. If the Mallory technique and the iron haematoxylin technique reveal selectively the same types of nuclei, the ratios of the two types must be identical in preparations treated in the same way, but stained by the two methods. Hence comparative counts must differ only to an extent controlled by the degree of accuracy of the sampling technique. In order to verify this hypothesis, material was selected to cover a wide variation of the relative proportion of the two types of nuclei (from 0 per cent. to 43 per cent. 'yellow' nuclei); counts were made on parallel preparations stained, one by the Mallory and the other by the iron haematoxylin technique.

The statistical analysis was made by using the χ^2 test applied to a test of independence in a fourfold table. Table 1 summarizes the results of a series of determinations. The nuclei counted in modified Mallory preparations are classified as 'yellow' (y) or 'blue' (b), those in iron haematoxylin preparations

as 'dark' (*d*) and 'light' (*l*). The values of χ^2 have been calculated from the abbreviated formula:

$$\chi^2 = \frac{(yl - bd)^2(y + b + d + l)}{(y + b)(d + l)(y + d)(b + l)}.$$

The individual values found for χ^2 must be compared with the distribution of probability of χ^2 for 1 degree of freedom. From the table of Fisher (1950), the limit of probability $P = 5$ per cent. for 1 degree of freedom corresponds to the value of $\chi^2 = 3.84$. From the inspection of the results it is obvious that no value found for χ^2 is superior to this limit. It may be concluded that the distribution of the cell types is identical with the two types of staining.

This result is reinforced by the combination of all the χ^2 tests.

It is known that the sum of a number of value of χ^2 follows the distribution of χ^2 , with the appropriate number of degrees of freedom. Entering the table of the distribution of χ^2 with 5 degrees of freedom, the value $\chi^2 = 4.008$, obtained in pooling the individual values of χ^2 , corresponds to a probability $P = 0.55$, far above the critical limit of $P = 0.05$.

TABLE I

Nuclear counts in preparations stained by Mallory's stain and iron haematoxylin

Prep. no.	Number yellow	Number blue	Number dark	Number light	Per cent. yellow	Per cent. dark	χ^2
A	0	..	0	..	0	0	..
B	100	519	100	671	16.2	13.0	1.733
C	236	322	229	311	42.2	42.4	0.0014
D	210	347	201	395	37.7	33.7	1.980
E	233	300	292	354	43.7	45.2	0.261
F	166	385	177	401	30.1	30.6	0.033
TOTAL	4.008

The mere fact that there is such good correspondence between the results obtained with the two staining methods demonstrates that the factors involved in the staining (concentration of the solution, duration of the staining and of destaining) are unimportant in determining the relative numbers of the two types of nuclei, and that the results are conditioned by factors inherent in the nuclei themselves.

Influence of fixation

The fixative recommended by Sheinin and Davenport is of the chromic acid mercuric type. Parr and others used perfusion with 10 per cent. formalin gum acacia, followed by the completion of fixation in 10 per cent. formalin. It appeared interesting to find whether the possibility of differentiating the two types of nuclei is related to a particular type of fixation. With the nine fixatives used it was possible to differentiate the nuclei either by the modified Mallory technique or by the iron haematoxylin technique. However, the contrast between the two types of nuclei was found to be very different

according to the fixative used, so that for practical purposes some of them appear unsuitable.

With both staining techniques, good contrast was observed in liver blocks fixed in Carnoy's and Bouin's fluids. The best results, however, were obtained after fixation in alcohol formalin and in alcohol formalin acetic acid.

Contrast is good in buffered formalin at either pH 3.5 or pH 7.0, but the preparations suffer from poor general fixation and lack of homogeneity.

Fluids containing mercuric chloride gave greatly inferior results. In Mallory preparations the staining of the 'yellow' nuclei is good after fixation in the acid mercuric chloride fluids (Zenker, Susa), but the blue staining of the 'blue' nuclei is strongly depressed. In Helly, this is reversed. In both cases the preparations lack contrast. In iron haematoxylin preparations, tolerable contrast was observed after Susa fixation. The general staining of the nuclei is very poor after Helly fixation, and differences between nuclei can hardly be seen. The very strong staining of the cytoplasm after Zenker fixation prohibits the study of the nuclei.

In conclusion, the same basic differences between nuclei may be observed after all the fixatives used. However, some fixing fluids interfere with the general staining properties of the tissues, so that the differential staining of the nuclei may be obscured.

Integrity of the nuclear membrane as the factor for the differential staining of nuclei

The study of sections of different thicknesses mounted on the same slide showed extraordinary differences between the proportion of the differently coloured nuclei. In sections at $4\ \mu$, no 'yellow' or 'dark' nucleus could be seen; in sections at $6\ \mu$, a few were present; in thicker sections, their number increased rapidly with increasing thickness. This led me to try to find out in what position in the section the nuclei are situated which are differentiated by the staining. Parr, Mossberg, Rosenzweig, Breslar, and Clark observed that differently coloured nuclei may lie at the same depth in the section. This is undoubtedly true. However, careful inspection of the preparations revealed the following fact: those nuclei that stain 'yellow' in Mallory preparations or 'dark' with iron haematoxylin are those that are completely included in the section, with *the nuclear membrane intact*. When the nuclear membrane is divided by the razor, so that a part of the nucleus is cut away, the nucleus stains 'blue' in Mallory preparations, or 'light' in iron haematoxylin preparations.

Fig. 1 shows three untouched photomicrographs of the same field taken at different levels, under oil-immersion lens, in a very thick ($16\ \mu$) section of liver stained with iron haematoxylin. In the upper level of the section (fig. 1, A), four nuclei of the 'light' type are focused. Microscopic examination showed that none remained untouched by the razor. The 'dark' nuclei present in deeper layers of the section are seen as spots with vague outlines. In B, taken in the mid-part of the section, ten 'dark' nuclei may be seen. Only one

'light' nucleus is present, which is actually the upper part of an incomplete, more deeply situated nucleus. In c, taken at the deepest level of the section, the 'dark' nuclei are out of focus; three 'light' nuclei are visible, their membranes having been partly cut away. These photographs show that only intact nuclei are able to stain 'dark'.

This circumstance explains the fact that 'dark' or 'yellow' nuclei are much more numerous in thick sections than in thin ones. The probability that a nucleus will be intact increases with increasing thickness of section. For a

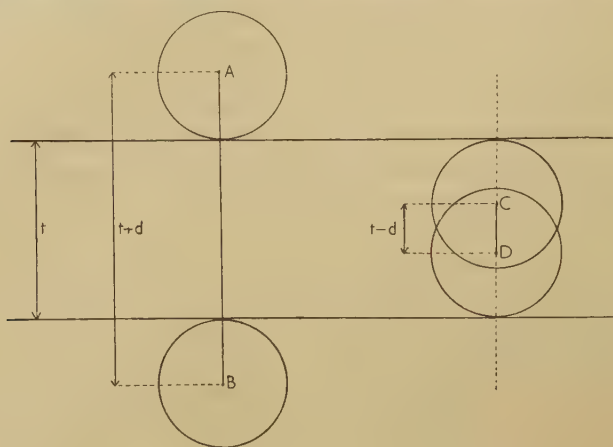


FIG. 2. In a section of thickness t , a nucleus of diameter d is contained in the section (left in the figure) if its centre occupies a position between A and B; this corresponds to a distance $t+d$. The same nucleus (right in the figure) is contained *entirely* in the section if its centre is located between C and D; this corresponds to a distance $t-d$.

given diameter of the nucleus this probability may be calculated. If t is the thickness of the section and d the diameter of the nucleus, the centre of a nucleus which is included (partially or entirely) in the section may occupy all the positions along a line whose length is $t+d$ (see fig. 2). This nucleus will be included *entirely* in the section if its centre falls on that part of the line which is marked C-D and whose length is $t-d$. Hence the probability p of a nucleus being included entirely in the section is:

$$p = \frac{t-d}{t+d}, \quad (1)$$

FIG. 1 (plate). A, liver of rat stained with Heidenhain's iron haematoxylin. Section 16μ thick. The focus is on the upper plane of the section. The nuclei located near the surface of the section are partly cut and are stained 'light'. The nuclei lying in deeper layers are out of focus and are seen as ill-defined dark spots.

B, same preparation as A, but the focus is on the central part of the section. In this layer the nuclear membranes are untouched, and for this reason the nuclei stain 'dark'. Only one nucleus in this figure is of the 'light' type; this one is lying in a deeper layer, as may be seen in c. Microscopical examination showed that the membrane of this nucleus has been partially cut away.

C, same preparation as A and B, but the focus is on the deepest layer. The 'dark' nuclei are out of focus. Three 'light' nuclei are visible, all of them with the membrane partly cut away.

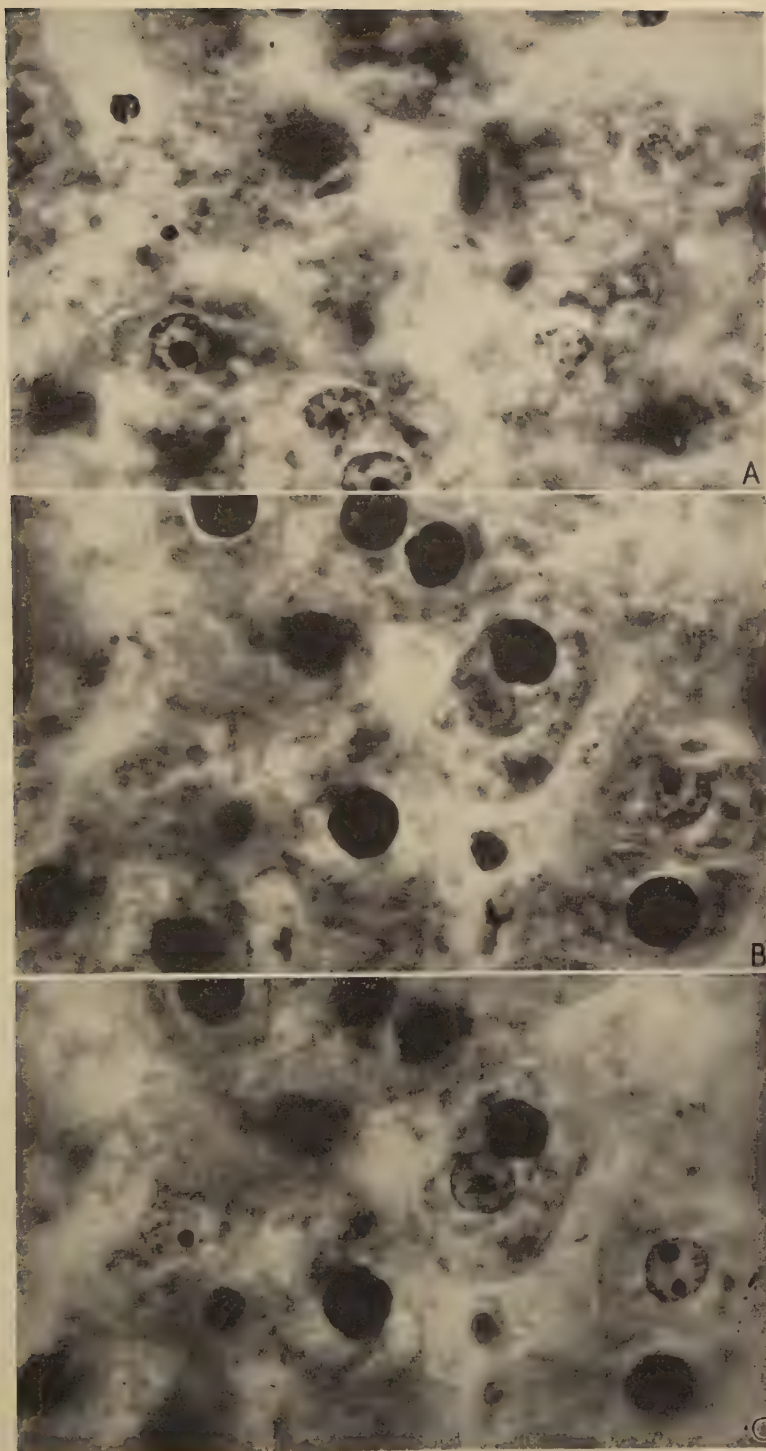


FIG. 1
L. LISON

and the probability of not being included entirely is, of course:

$$q = 1 - p \quad \text{or} \quad q = \frac{2d}{t+d}. \quad (2)$$

If the staining method is able to differentiate the untouched nuclei from those which are cut, differential counts in sections of various thicknesses must give ratios identical to these probabilities.

In order to verify this hypothesis, counts were made in sections of the same liver, made at 4, 6, 8, 10, 12, and 14 μ , mounted on the same slides and stained with the modified Mallory method. The results are expressed in fig. 3, which gives the percentage of the 'yellow' cells found in the preparations.

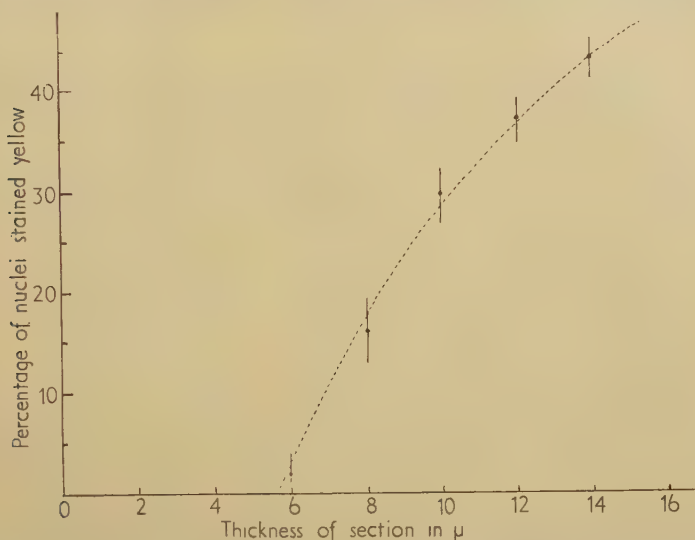


FIG. 3. Results of differential counts of nuclei in sections of the same rat liver stained by the modified Mallory stain. The percentage of nuclei stained yellow is plotted as a function of the thickness of the section. The broken line represents the theoretical percentage of the 'yellow' nuclei calculated from the regression equation (4). The points indicate the values found. The upper and lower limits of the confidence-interval for a probability $P = 0.05$ are indicated as vertical lines. (Note the asymmetry of the confidence-interval for the lowest percentages.)

The vertical lines indicate the upper and lower limits of the confidence-interval at a probability level of 5 per cent. The broken line represents the theoretical values calculated as indicated hereafter.

The above expressions (1) and (2) for p and q do not suit very well for the computations. However, if we let $Y = p/q$, then we obtain

$$Y = \frac{t-d}{2d} \quad \text{or} \quad Y = \frac{t}{2d} - 0.5. \quad (3)$$

When studying the same liver, d is constant and Y is a straight-line function of the thickness of the section. The best fit for the parameters of the equation

(3), which represents the regression of t on Y , may easily be calculated from the experimental data by the least squares method. This was done for the data obtained, with the following result:

$$Y = 0.0928t - 0.529. \quad (4)$$

It may be verified that the constant term 0.529 is only slightly different from the theoretical value, 0.500. From the values of Y calculated from equation (4) the percentages expected for the 'yellow' or 'dark' nuclei were calculated from the formula: $p = \frac{Y}{1+Y}$. The comparison of the values found with the values expected from the theoretical data is given in the following table.

TABLE 2

Theoretical and experimental values of the regression: percentage of 'yellow' or 'dark' nuclei / thickness of the preparation

Thickness in μ	Y found	Percentage calculated	Percentage found, with standard deviation
		%	%
4	-0.158	0	0
6	0.02765	2.7	2.0 ± 0.6
8	0.2192	17.6	16.2 ± 1.3
10	0.3987	28.5	30.1 ± 1.9
12	0.5843	36.9	37.7 ± 2.0
14	0.7698	43.5	43.7 ± 2.1

As the table shows, the fit between the experimental data and the expected values is as good as can be expected.

I conclude that both histological evidence and statistical analysis demonstrate that the integrity of the nuclear membrane is the factor causing differential staining of the nuclei.

Differential staining of nuclei in other organs than liver

Sheinin and Davenport, and Parr and his associates, described the differential staining of nuclei only in the parenchymal liver cells. I investigated a variety of other organs by the same methods. I found that the nuclei of some cell-types are able to give a differential stain in the same conditions as in the liver, when other cell-types, sometimes in the same organ, do not show any differences at all. Table 3 summarizes these results.

DISCUSSION

My findings are contrary to the view expressed by Sheinin and Davenport, and by Parr and others, that there exist two types of nuclei in the parenchyma of the liver which differ by some histochemical character or which represent nuclei in different physiological states. I found that it was possible to stain

the nuclei differentially with Mallory's stain, and demonstrated that Heidenhain's iron haematoxylin allows the same differentiation. However, it has been shown by the use of appropriate techniques that the factor responsible for the differential staining is the integrity of the nuclear membrane. When the membrane is intact, the nucleus stains 'blue' with the modified Mallory method, or 'light' with the Heidenhain iron haematoxylin; when it is partially cut away, the nucleus stains 'yellow' or 'dark'. This phenomenon is not restricted to the liver cells, but is shown also by a number of other cell types, but not by all.

TABLE 3
Occurrence of differently stained nuclei in different rat organs

pancreas	acinar cells	+	kidney	glomerular epithelium	+
	pancreatic ducts	—		proximal convoluted segment	+
	islets of Langerhans	—		loop of Henle	—
thymus and lymph nodes	lymphocytes	—	ureter	distal convoluted segment	—
	reticular cells	+		collecting tubules	—
	macrophages	+		mucous membrane	+
testis	all cells except spermatozoa	—	supra-renal	cortex	+
		+		medulla	—
seminal vesicles	epithelial cells	+	submaxillary gland	tubular segments	+
				acinar segments	+
				excretory ducts	+
connective tissue	fibrocytes	+	sublingual gland	all parts	+
	adipose cells	+			
+ Nuclei differently stained			— All nuclei similarly stained		

I am not able to explain entirely the mechanism of the differential staining. However, there is some evidence that the main factor may be the relatively low permeability of the nuclear membrane. This is strongly suggested by a consideration of the mechanism of the staining methods which are able to give a differential stain of the nuclei.

In the Heidenhain's iron haematoxylin method, a highly insoluble dye-lake is formed in the tissue by successive prolonged treatment with iron alum and haematoxylin solutions. During differentiation the dye-lake is slowly extracted by further treatment with iron alum, in which the lake is soluble. It may be admitted that the relatively large dye-lake molecule does not pass readily through the nuclear membrane during the destaining. If the membrane is cut away, the solution of the lake under the action of the iron alum is much more rapid. For this reason, the nuclei with intact membranes are much darker than the other ones.

The Mallory method makes use of a mixture of the acid dyes orange G and aniline blue. Von Möllendorff demonstrated long ago (1924) that aniline blue is much less diffusible in protein gels than orange G. When one uses a mixture of the two dyes, the penetration of the less diffusible stain through membranes of low permeability is likely to be much less than the penetration of the more diffusible. When the nuclear membrane is intact, the orange G is the only dye of the mixture that is able to pass readily through the membrane. The orange G acts as if it were alone and stains those nuclei yellow. When the nuclear membrane is partially cut away, both stains enter the nuclei and compete for the staining of the nuclear components. As aniline blue is more adsorbable than orange G, it excludes the latter to a large extent, so that the nuclei appear blue.

It may seem rather strange to speak of the permeability of the nuclear membrane in dead cells after histological fixation, for it appears questionable whether a killed cell can retain any selective permeability. However, the suggested differences of permeability are not necessarily of the same selective type as during life.

The fact that some types of nuclei do not give any differential stain is interesting, for it suggests that the properties of the nuclear membrane in the fixed state may vary according to the cell-type.

In the experiences of Parr and others, changes in the proportion of the two types of nuclei were observed when rats were submitted to starvation or to a protein-free diet. It is very doubtful whether this change represents actual modification of the chemical composition of the nuclei. It results from theoretical evidence and from my experimental studies that the proportion between the differentially stained nuclei depends upon the thickness of the sections and upon the diameters of the nuclei. It is to be expected that any treatment capable of modifying the volume of the nucleus will change this proportion. For this reason it is believed that the action of the starvation or of a protein-free diet is explained by modifications in the nuclear volume. In the counts of Parr and others striking differences were observed between the means of the various untreated control groups. Three series of experiments gave the following values for the mean percentage of 'blue' cells in normal animals: 94.7 per cent., 84.4 per cent., and 61.2 per cent. The authors speculated about the explanation of such extreme variations. They suggested (without experimental proof) either genetic differences between the animals used in the experiments, or 'slight, and perhaps unavoidable' variations in staining and differentiation of the slides. My opinion is that the three series of counts were made either on sections of varying thickness or on blocks embedded in paraffin under different conditions, with the result that the unavoidable shrinkage of the tissues during the embedding process was different.

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The Structure and Connexions of the Corpora Pedunculata in Bees and Ants

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With two plates (figs. 3 and 6)

SUMMARY

1. A method is described of using high frequency radio waves to produce localized and precisely controlled burns. The technique is employed to damage the cells of the corpora pedunculata in bees and ants, and to follow the subsequent degeneration in these lobes.

2. The corpora pedunculata on each side of the brain are composed of four lobes: two calyces, an α lobe and a β lobe. Each fibre originates from a cell in the calyx, and has three branches, one to the calyx itself, one to the α lobe, and one to the β lobe. The arrangement of the tracts in these lobes is described.

3. The calyces and the α lobe both receive tracts from all the sensory centres of the brain, while the β lobe sends tracts to the motor regions. The detailed connexions of these tracts are described.

4. It is suggested that in the functioning of the corpora pedunculata the excitations from the calyx and the α lobe will interact, and that the excitation passing away down the β lobe is the result of this interaction.

INTRODUCTION

DURING the past two years a physiological study has been made of the corpora pedunculata (mushroom bodies) in bees and ants. The superficial position of the cell-bodies in the central nervous system facilitated this study, which involved localized damage to the cells of the brain, followed by examination of subsequent degeneration. It is believed that the application of such a technique to invertebrates has seldom been attempted, and one object of this paper is to draw attention to the possibilities of the method. The use of this technique has enabled various assertions made by previous authors, on the basis of silver staining alone, to be re-examined.

Since the description of the 'corps pédonculés' by Dujardin (1850) these lobes have attracted much attention from microscopists. Papers by Kenyon (1896), Jonescu (1909), and Sanchez (1941) on the honey bee, and by Pietschker (1911) on the ant, are perhaps the most important contributions for the Hymenoptera. Thompson (1913) described various tracts within the mushroom bodies of ants, but the present study has failed to confirm many of her assertions, owing perhaps to her use of haematoxylin, which does not lend itself to detailed neurological studies. Hanström (1928) gives a survey of the older literature.

All these descriptions of the corpora pedunculata fall into two main groups: those dealing with the gross anatomy of the organ, and those concerned with minute details of individual neuron connexions. Between these two extremes lies what may be called the functional anatomy of the organ, dealing with a level of organization intermediate between the general form and the fine

detail. It is knowledge of precisely this level which was required in the present physiological study. This knowledge was unfortunately lacking, and, indeed, the detailed descriptions of the mushroom bodies already available, while important for some purposes, have tended to obscure the fundamental simplicity of their organization. The present paper attempts to remedy the deficiency and to give an account of the functional anatomy of the corpora pedunculata in bees and ants.

MATERIAL AND METHODS

The brain of the honey bee, *Apis mellifica*, and of the ants *Camponotus pennsylvanicus*, *Formica rufa*, *Eciton hamatum*, *Paraponera clavata*, and *Cryptocerus angulosus*, were examined; only the media workers were studied, and no attempt to compare either castes or species has been made. All the brains were stained by the silver-pyridine technique developed by Holmes (1947), which gave a consistently good result both for tracts and for individual axons. Two fixatives were found to be suitable: mercuric formaldehyde gave a good, rather bluish, stain with ants, while alcoholic Bouin gave a good reddish stain with bees.

The insects to be fixed were first anaesthetized with carbon dioxide, while the chitin overlying the brain was removed: this was found to be necessary to allow adequate penetration by the fixative. The toughness of the chitin precluded the regular preparation of a complete series of sections of the whole head, and the fixed brains were therefore dissected out before being embedded in the usual way.

In the experiments, in which the brains were intentionally damaged, two types of cautery were used. The first type was simply a nickel needle, of suitable size, heated to a dull red in the flame from a spirit lamp, and then placed immediately against the surface of the brain. With practice this gave fairly good results, and the method has the advantage of simplicity; there were, however, two serious disadvantages, both the result of lack of control of the heating effect: firstly, if the needle was too hot it seared the tissue for an ill-defined area around the point of contact, and, secondly, if the needle was too cold no damage was caused, and the operation was wasted.

It was obvious that what was required was a cautery, the power of which could be varied, and which would damage a well-defined and precisely controlled area. Such a cautery was constructed by utilizing the property of high frequency radio waves to produce burns when concentrated in a small area, while not harming a tissue in which they are dispersed. It is not proposed here to give details of the R.F. cautery which was constructed, for the same output could be achieved by a variety of different arrangements, all equally satisfactory, and indeed it is hoped to improve on the present apparatus. The output available was as follows:

Frequency: 1.97 megacycles.

Max. power: 25 volts.

Min. power: 9 volts.

The most variable factor is the contact between the electrode and the tissue; a close, firm contact is essential for a good result. With the maximum power the damage produced made a small hole extending down from the electrode into the brain; with the minimum power the effect was only to coagulate the protoplasm of the cells with which the electrode made contact. The minimum power was therefore used when operating on the calyx, while the maximum power was employed in cauterizing the α lobe (see later).

The electrodes used were of platinum wire, insulated to the tip with glass. They were prepared by the method given by Johnson and Manhoff (1951). The area of damage to the brain coincided with the area of contact with the electrode; the diameter of the wire was therefore selected according to the amount of damage intended. For the success of the operation it was necessary that the insect should have a large capacity to earth: this was obtained by placing the abdomen in contact with an earthed metal plate.

The operating table was made of soft plasticine, moulded into a groove to fit the animal's body; the insect, with its head exposed, was then covered by another layer of plasticine over the thorax and abdomen. The bee or ant was thus held firmly by the plasticine which was, however, sufficiently yielding to prevent damage to the body or its appendages.

A series of exploratory dissections was done, and the various parts of the brain were localized with respect to various external features of the head. An experimental bee or ant was anaesthetized with carbon dioxide and placed on the operating table, which was set up on the stage of a dissecting microscope. A slit was made in the chitin of the head over the region to be cauterized. The electrode was then inserted until it was in contact with the surface of the brain, and the power switched on for 2–3 seconds; if a successful contact had been made the antennae usually twitched when the current was first switched on. The electrode was then removed and the slit in the head closed by flowing dental wax over the surface; this wax was eminently suitable for the purpose as it had a low melting-point, could be moulded easily with a warm needle, and formed a good joint with chitin. The operated insect was marked with cellulose paint for identification, and kept alive for several days. Degeneration was usually complete after 3 days from the time of operation. The brain was then fixed and sectioned as described.

A series of operations was done, both with bees and with all the species of ant listed. In the initial series the mushroom bodies on one side of the brain were completely destroyed, while in subsequent series the area of damage was progressively reduced until only small portions of the calyx were injured. The relatively small injuries were more easily produced on the bee's brain than on the ant's, owing to the larger size of the former, and for this reason the mushroom bodies of the bee have been studied in the more detail. So far, the damage has been restricted to the mushroom bodies themselves, but it is hoped to extend the study to other tracts and lobes of the brain. The use of electrodes insulated to the tip raises the possibility of causing damage deep in the brain—a technique which should give interesting results.

The general form of the corpora pedunculata

The corpora pedunculata in bees and ants are paired structures lying symmetrically on either side of the midline of the brain, and occupying the bulk of the protocerebrum. The structure on each side consists of a pair of fibrous calyces, which occupy the whole posteriodorsal region of the brain between the midline and the optic ganglia, and from which tracts descend to other regions of the brain. The rim of each calyx is roughly oval in shape: the medial calyx extends the further forwards, and overlaps the lateral calyx on the frontal surface of the brain, the condition being reversed on the lower

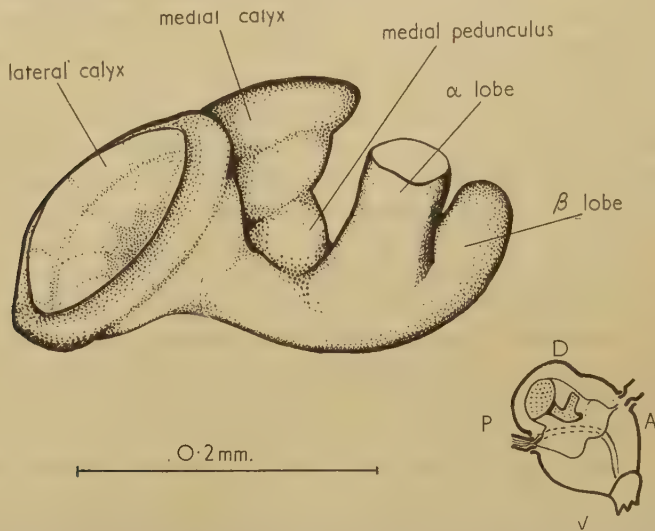


FIG. 1. A reconstruction of the corpora pedunculata of the right side of the brain of *Formica rufa* (worker), seen from the antero-lateral aspect. *Inset*: the head of the ant from the right side, showing the position of the brain and the corpora pedunculata (stippled).

surface where the lateral calyx extends further back. The cell bodies of the corpora pedunculata fill the calyces and slightly 'overflow' them. The rest of the structure is fibrous in nature and fairly sharply delimited from the rest of the brain tissue.

From each of the two calyces a short stalk, or pedunculus, extends downwards and forwards into the protocerebrum; the two stalks fuse with each other after a short distance and immediately after the point of fusion two other lobes are formed: the first of these passes forwards and upwards to the frontal surface of the brain, where it ends abruptly as a flat disk beneath the two or three layers of cell bodies which cover this region of the protocerebrum; the second lobe turns downwards and inwards and ends abruptly on the deep midline of the brain, where it abuts against the similar structure from the other side. The three lobes will here be referred to as the calyx, the α lobe, and the β lobe, as shown in fig. 1. (See discussion below.)

All the nerve fibres constituting the corpora pedunculata originate from

cells within the bowl of the calyx. Each fibre has three branches, one to each of the three lobes, where they make synaptic connexions with incoming fibres from other parts of the brain. The calyx and the α lobe receive fibres from sensory centres, while the β lobe gives connexions to fibres going to motor centres (see later).

The names of the various lobes of the corpora pedunculata have suffered from much confused variation. The terms 'calyx' and 'pedunculus' which are apt when applied to the Hymenoptera, and have the authority of long usage, will be used here; they would not be applicable to such orders as the Diptera, in which the equivalent of the calyx is a mere aggregation of cells (Bretschneider 1913; Power 1943). The other lobes have usually been referred to in English as 'roots', e.g. the anterior root and inner root of Kenyon (1896). The use of these terms has two disadvantages:

1. The word 'root' implies in its general sense both a source, or origin, and a means of attachment. In neither of these senses can the term be applied to the corpora pedunculata, for the fibres of this organ all originate in the calyx, and all end within the other lobes, making it a discrete structure, which is attached to the rest of the brain only by tracts originating in other parts of the brain, and most of which come to the calyx.

2. The word 'root' is used in a specifically neural sense to designate those parts of peripheral, segmental nerves which lie immediately outside the central nerve cord. The 'roots' of the corpora pedunculata in no way resemble these.

Power (1943) has used the term 'stalk' in preference to 'root', following some other authors, but this does not resolve the problem, and indeed he is forced into the paradoxical situation of referring to the same structure both as a root and a stalk. It would seem preferable to avoid the use of botanical terms altogether, as Hanström (1928) and some other German authors may have tried to do by employing the terms 'rückläufiger Stiel' and 'Balken': these terms still have some disadvantages, however, for in some insects, e.g. *Drosophila*, the 'Balken' appears more recurrent than the 'rückläufiger Stiel'. It is also difficult to give a precise English translation of these two terms.

The three main subdivisions of the corpora pedunculata are in fact lobes, each with its own function, and it would seem desirable to refer to them as such. The lobes could then be further distinguished by reference to their relative positions, e.g. the upper lobe and the lower lobe, or the anterior and the posterior lobe. This would be unwise, however, for in different insects the apparently homologous lobes occupy very different positions: in the Lepidoptera and Hymenoptera, for example, the two homologous lobes (α lobe) run posteriorly and anteriorly respectively. In our present state of knowledge, therefore, it is preferable to give the lobes only general names which can be more precisely defined by future work in this field.

In the present work it is proposed to refer to the two lobes as the α lobe and the β lobe respectively, as shown in fig. 1. This is preferred as the terms are indifferent as to structure, function, and orientation; thus one avoids confusion in comparative studies of orders in which the homologous lobes may

differ in all three factors. At the same time the general nature of the terms leaves the field clear for more precise definition in the future.

The relationships of the corpora pedunculata to the other lobes of the brain are shown in fig. 2. The relative sizes of the corpora pedunculata differ in different species of ants, being smallest in *Eciton hamatum* and largest in *Formica rufa*; this may be related to the fact that the eyes and optic ganglia

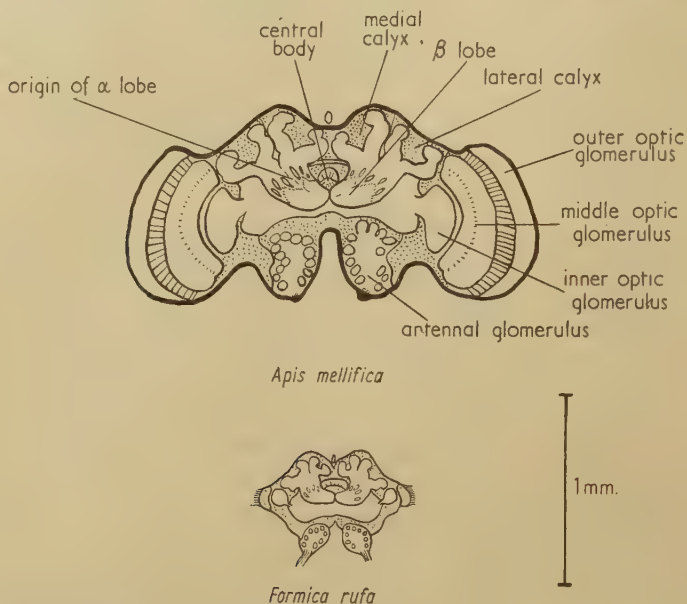


FIG. 2. Frontal sections of the brains of the honey bee (*Apis mellifica*) and the Wood ant (*Formica rufa*).

are small and degenerate in the Army ant, but large and well developed in the Wood ant (Werringloer, 1932). Goossens (1951), like other workers, has shown that the sizes of the mushroom bodies and the central body are inversely proportional, a fact which he attributes to the limited space available in the brain.

The fibres within the mushroom bodies are of two types, those originating within the organ and those arising from other association cells and connecting the mushroom bodies with the sensory and motor lobes of the brain. The fibres originating within the organ all arise from the cell-bodies in the calyces. According to Kenyon (1896) these neurons are all of one basic type: from each cell-body a projection passes down into the calyx where it divides into two parts, one forming arborescent synaptic connexions within the calyx, and

FIG. 3 (plate). A series of frontal sections of the brain of *Apis mellifica*. The outer half of the lateral calyx has been cauterized, and the degeneration can be seen spreading through the lobes of the corpora pedunculata. Only the damaged half of the brain is shown in full. A, the 6th section, counting from the frontal surface of the brain; B, the 16th; C, the 19th; D, the 26th.

The sections were cut at $15\ \mu$ and stained with Holmes's silver stain.

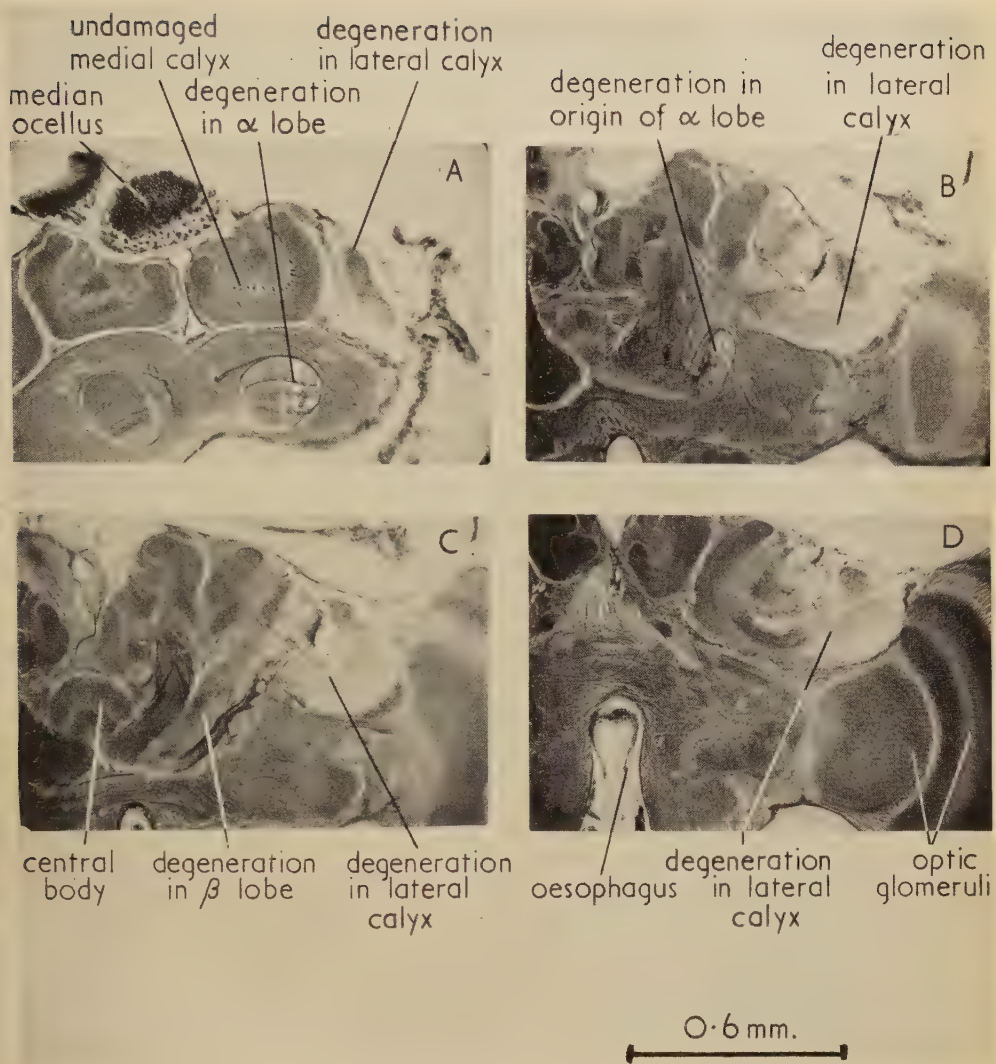


FIG. 3
D. M. VOWLES

the other passing on down the pedunculus until it reaches the origin of the α lobe, where it bifurcates, sending one branch up this lobe and the other down the β lobe, making synaptic connexions in both regions. The course of these fibres, provisionally established in silver preparations of normal material, has been confirmed in the present work, from the study of their degeneration. In fig. 3 is shown a series of sections of a bee's brain in which the lateral half of the lateral calyx has been cauterized; the degeneration can be seen passing from the calyx, down the pedunculus, and along the whole length of both the α and β lobes. Detailed examination of such preparations reveals that the amount of degeneration remains fairly constant at all levels of the pedunculus and the lobes, which suggests that all the fibres extend for the whole distance. After leaving the calyx the degeneration also preserves its position relative to the circumference of each lobe, showing that the fibres do not deviate from their approximately straight course. The degeneration always ends within the mushroom body itself, which suggests that the fibres do not pass out into the mass of the brain; this should be particularly noted in reference to Thompson's (1913) assertion that the tubercles of the central body are continuations of the β lobe, for the degeneration never extends into these tubercles, neither does the degeneration spread from one calyx into its neighbour, although Thompson also claimed that fibres take this course.

The association fibres from other parts of the brain pass in at the base of each calyx, and all round the sides of the α lobe and β lobe. These fibres do not degenerate together with those of the mushroom body; in fig. 6, B can be seen fibres from the optic ganglia passing through a degenerated region of the α lobe.

Tracts within the calyx

Most previous workers have noticed that the cells in the central region of each calyx stain more deeply with haematoxylin and silver than do the peripheral cells. This has been confirmed in the present work, and the appearance of these cells is shown in figs. 3, 4, and 6, c. Thompson claimed that in *Camponotus pennsylvannicus* she could identify several groups of cells of different sizes across a single section of a calyx; these groups of cells have not been distinguished in my preparations. Fibres from the cells of the calyx are very fine, and have not stained individually or distinctly, except in a few cases. The fibres entering the corpora pedunculata, on the contrary, are fairly coarse and have stained clearly and precisely. In neither case, however, have fine terminations been seen, and it appears that Holmes's stain is not suitable for such regions of these nerves. It is assumed that the synapses are of the arborescent type as described by Kenyon (1896) and confirmed by Sanchez (1941).

The fibres from the cell-bodies in the calyx pass directly outwards into the calyx wall. The fibres from the central group of cells stain more deeply than those from peripheral cells, and can be seen lying in a compact mass which forms a ring around the centre of the calyx. Degeneration studies show

that fibres run for a considerable distance around this 'central ring', often passing around to a point diametrically opposite to their place of entry. This is unlike the fibres from the peripheral cells, which do not pass far around the calyx.

The descending fibres from the peripheral cells are gathered into a series of radially arranged bundles, which become sharply defined as they pass downwards, and then turn horizontally inwards over the central ring, fuse with each other in the centre, and pass directly down through the ring into the pedunculus. Fibres from the central ring are not gathered into bundles,

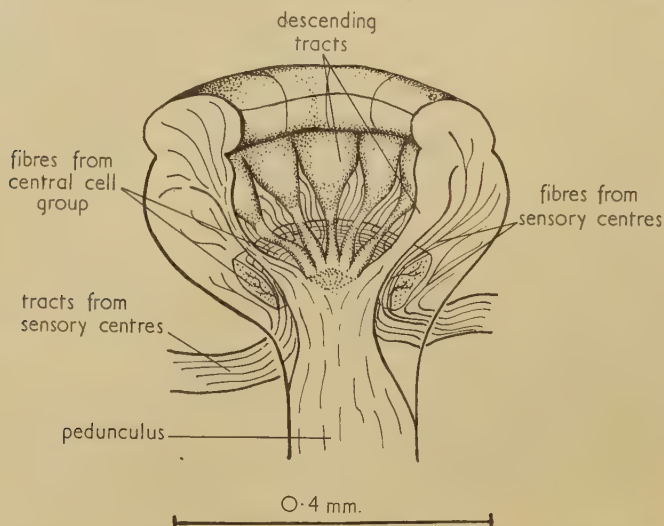


FIG. 4. A diagram to show the arrangement of tracts within the calyx of *Apis mellifica*.

but pass directly inwards to the middle of the pedunculus and then turn down it.

The association fibres which enter the calyx from other parts of the brain do so at the junction of the calyx and the pedunculus. They then pass upwards, both inside and outside the central ring, to which they remain closely apposed, and pass around the calyx below the descending bundles, giving off branches into the peripheral region of the calyx and into the central ring. These afferent fibres form synaptic connexions with the fibres of the calyx itself, and Kenyon has recorded that a single afferent fibre may have connexions with many different calyx fibres.

Tracts within the α lobe

There are no separate tracts within the α lobe. The fibres coming from the pedunculus run straight along the lobe to its termination, and in sections transverse to its long axis it has a fairly homogeneous appearance. Association fibres from the rest of the brain enter over the whole of the surface, except at the extreme frontal tip. Tracts of association fibres which reach the surface immediately spread out and run around the lobe in both directions, sometimes

almost completely circumscribing it, and resembling in appearance a current of water eddying and swirling round a rock. Individual fibres are thus enabled to enter the lobe all around its surface; once inside they branch fairly profusely in all directions and run through the whole substance of the lobe, forming synaptic connexions with the mushroom body fibres. A typical

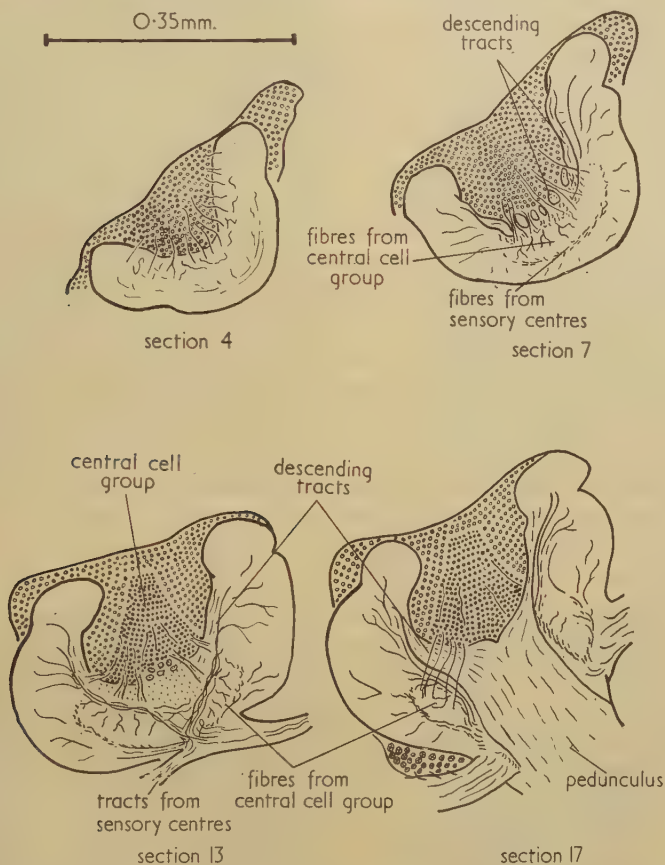


FIG. 5. Drawings of a calyx of *Apis mellifica* as it appears in a series of frontal sections of the brain.

arborescent fibre is shown in fig. 6, A. Sections at various levels of the lobe show a striated appearance, as in fig. 3, A. Kenyon claims that these striae are concentrations of synapses: if this is so then many association fibres must run along the length of the lobe for some distance, for at any one level the striae are restricted to one or two chords across the lobe, although the fibres spread out through the whole area.

Tracts within the β lobe

The appearance of the fibres within the β lobe is very similar to that described for the α lobe above. The tracts of association fibres coming to this

lobe are, however, much fewer in number (see later), more diffuse, and run along rather than around the surface of the lobe, see fig. 6, c.

The association fibres which run along the surface of the lobe turn into it, branch, and synapse with the mushroom body fibres. Striations can again be seen, but they lie in bands parallel to the long axis of the lobe, rather than across it.

The connexions of the corpora pedunculata

The fibres of the corpora pedunculata all originate and end within the organ itself, which therefore depends entirely upon tracts coming from and originating in other regions of the brain. These tracts are of two types: those coming from the sensory centres, which all go to either the calyx or the α lobe, and those going to motor centres from the β lobe. It is not proposed to describe the course of these numerous tracts in any detail, but merely to list them, together with a statement of their terminations. All the tracts will be referred to by numbers, and their correspondence with previously named tracts indicated where appropriate.

No detailed comparison of the insects studied has been attempted, for the amount of work involved would not be merited by the little we know either of true homologies in the insect brain or of the precise integrative functions of its lobes. The most notable difference between bees and ants is the large size of the optic glomeruli and their associated tracts in the former. Within the ants studied themselves the Army ant has degenerate optic glomeruli, and the tracts from them are insignificant. The brain of *Paraponera clavata* is very much larger than that of the other species, and is more easily studied, but the more common *Formica rufa* has been chosen for the type specimen here. However, the fundamental plan of the brain and its tracts is the same in all the species studied, which differ only in relative sizes and precise spatial relationships.

The numbers which have been given to the tracts indicate their order of appearance in frontal sections, starting with that nearest to the frontal surface of the protocerebrum. The size of the tracts has been exaggerated in the diagrams, and many of them (e.g. 6, 8, and 10 in fig. 7) have previously been referred to as bundles rather than as tracts; the latter term is used here for the sake of uniformity.

(A) *The tracts in the honey bee*

Tracts to the α lobe

1. Passing between the two α lobes. (Cell group I of Kenyon.)
2. From the inner optic glomerulus to the α lobe, and then across to the opposite side of the brain, where it has the same connexions.

FIG. 6 (plate). The fibres of various lobes of the corpora pedunculata of *Apis mellifica*.

A, section across an intact α lobe.

B, section across a partially degenerated α lobe.

C, section through a calyx, corresponding to fig. 5, section 7.

D, section through the upper surface of the β lobe.

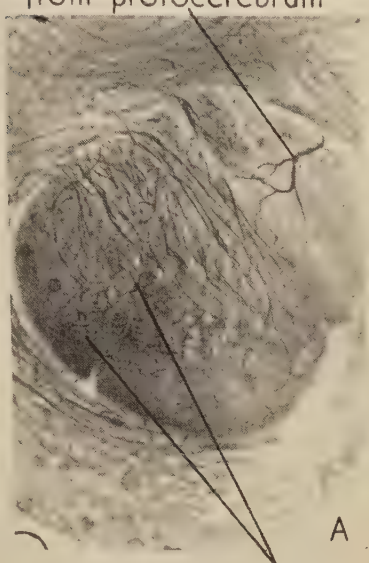
All the sections are frontal; in each case the dorsal side is turned towards the right side of the plate.

"central ring"



central cell group

arborescent neuron
entering α lobe
from protocerebrum

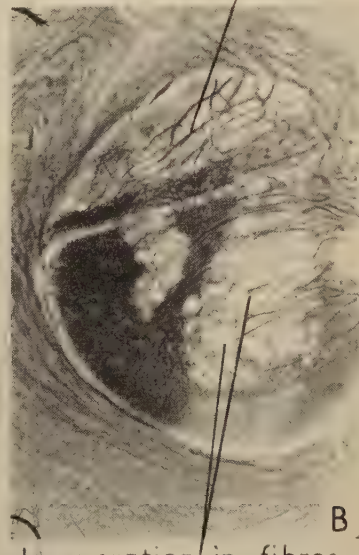


body of α lobe

undegenerated afferent fibres



fibres to central body



degeneration in fibres of
corpora pedunculata

0.18mm.

FIG. 6
D. M. VOWLES

3. From the optic tubercle to the α lobe. (Cell group V of Kenyon.)
4. From the inner optic glomerulus to the α lobe.
5. From the middle optic glomerulus to the α lobe. This tract has three origins: one from the middle region of the glomerulus and one each from the upper and lower surfaces.

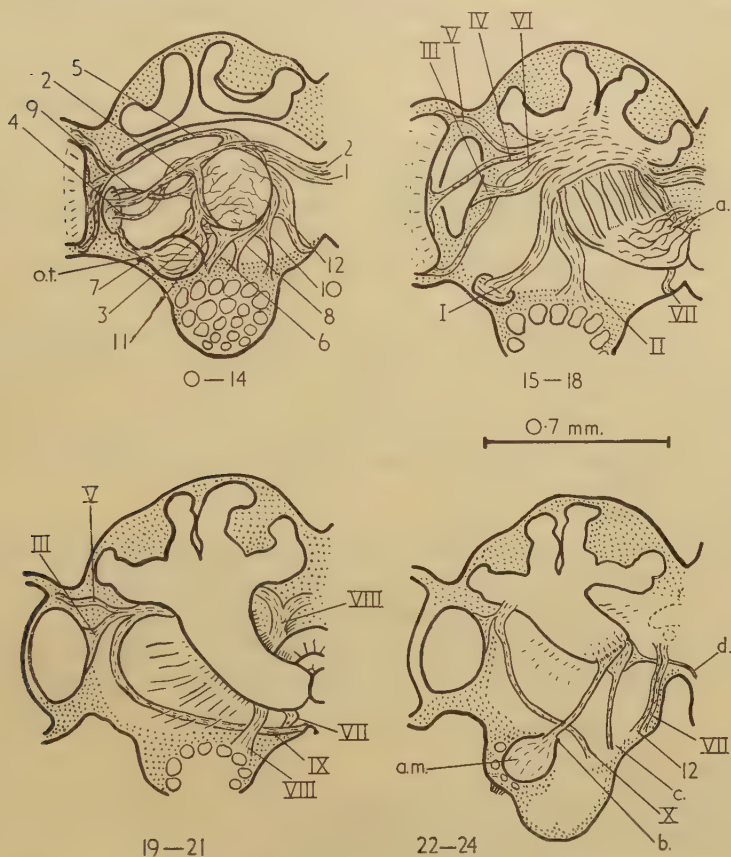


FIG. 7. Diagrams to show the tracts connecting the corpora pedunculata with the rest of the brain in *Apis mellifica*. The numbers of the frontal sections in which the tracts appear are shown below each picture. For description see text.

o.t. = optic tubercle.

a.m. = antenno-motor centre.

6. From the outer side of the antennal glomerulus to the α lobe. (Cell group VII of Kenyon.)
7. From the optic tubercle to the α lobe. (Cell group IV of Kenyon.)
8. From the middle inner surface of the antennal glomerulus to the α lobe. (Cell group VII of Kenyon.)
9. From the inner optic glomerulus to the α lobe.
10. From the middle inner surface of the antennal glomerulus to the α lobe. (Cell group VII of Kenyon.)

11. From the optic tubercle to the α lobe.
12. From the suboesophageal region to the α lobe.

Tracts to the calyces

- I. From the optic tubercle to the calyces.
- II. From the antennal glomerulus to the lateral calyx. (The outer antenno-cerebral tract of Kenyon.)
- III. From the inner optic glomerulus to the calyces.
- III'. From the inner optic glomerulus to the calyces, but passing below the pedunculi and originating lower in the optic glomerulus. (The postero-superior optic tract of Kenyon.)
- IV, V, and VI. From the middle optic glomerulus to the calyces. (The antero-superior optic tract of Kenyon.)
- VII. From the suboesophageal region to the calyces. (The dorso-ventral tract of Kenyon.)
- VIII. From the antennal glomerulus to the medial calyx. (The inner antenno-cerebral tract of Kenyon; the tractus olfactorio-globularis of Hanström.)
- IX. From the suboesophageal region of the opposite side to the lateral calyx.
- X. From the suboesophageal region of the same side to the lateral calyx.

Tracts from the β lobe

- a. Coarse fibres passing from the β lobe to the central complex beneath the central body. These are not gathered into a well-defined bundle.
- b. From the β lobe to the antenno-motor centre.
- c. From the β lobe to the suboesophageal region of the same side.
- d. From the β lobe to the suboesophageal region of the opposite side.

(B). The tracts in the ant *Formica rufa*

Tracts to the α lobe

1. Passing between the two α lobes.
2. From the inner optic glomerulus to the α lobe.
3. From the optic tubercle to the α lobe.
4. From the middle optic glomerulus to the α lobe.
5. From the suboesophageal region to the α lobe.
6. From the antennal glomerulus to the α lobe.

Tracts to the calyces

- I. From the optic tubercle to the calyces.
- II. From the antennal glomerulus to the lateral calyx.
- III. From the inner optic glomerulus to the calyces.
- IV. From the antennal glomerulus to the lateral calyx.
- V. From the middle optic glomerulus to the calyces.
- VI. From the suboesophageal region to the medial calyx.
- VII. From the suboesophageal region to the lateral calyx.

- VIII. From the antennal glomerulus to the medial calyx.
 IX. From the antennal glomerulus to the lateral calyx.
 X. From the antennal glomerulus to the lateral calyx.
 XI. From the suboesophageal region to the lateral calyx.
 XII. From the antennal glomerulus to the lateral calyx.

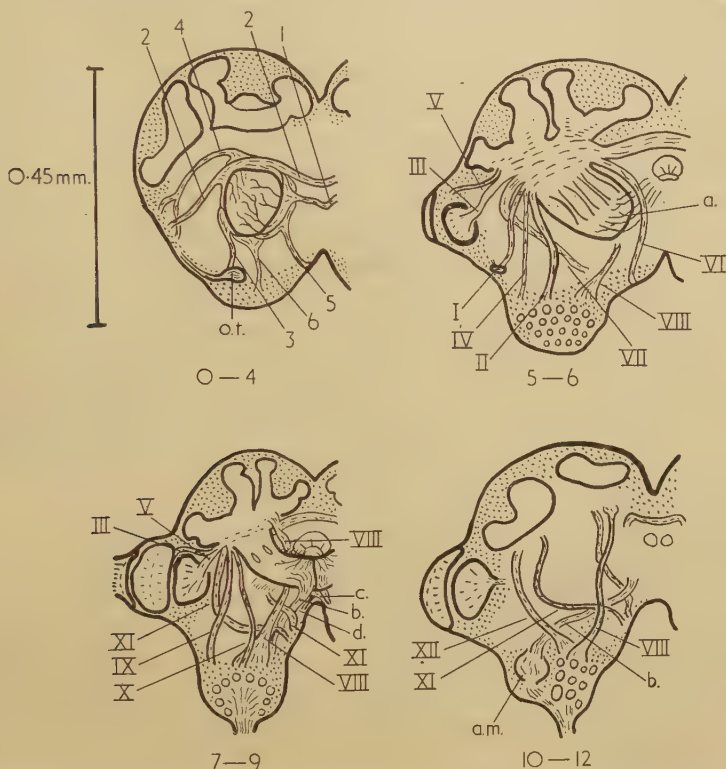


FIG. 8. Diagrams to show tracts connecting the corpora pedunculata with the rest of the brain in *Formica rufa*. The numbers of the frontal sections in which the tracts appear are shown below each picture. For description see text.

o.t. = optic tubercle.

a.m. = antenno-motor centre.

Tracts from the β lobe

- a. From the β lobe to the central complex, beneath the central body.
- b. From the β lobe to the antenno-motor centre.
- c. From the β lobe to the suboesophageal region of the opposite side.
- d. From the β lobe to the suboesophageal region of the same side.

In view of Power's (1946) criticism of Sanchez's (1941) term 'antenno-motor centre' it should be stated that the present study both confirms and extends the latter author's observations. Unlike the situation in *Drosophila* the major part of the antennal nerve does enter the antennal glomerulus in the

honey bee and in ants. The fibres which pass by the morula are of two sorts: numerous large, coarsely-staining motor fibres which pass on the ventro-lateral side of the morula to the fibrous structure called the antenno-motor centre; and much fewer lightly staining fibres which pass below the morula to its medial, inner side, and end in a few glomerular bodies resembling those of the true morula. In the above account the antennal glomerulus is taken to include both the morula-like lobes: their tracts can be distinguished from that to the motor centre. A commissure of the motor lobe has been distinguished passing just above the oesophageal foramen, and this implies that the lobes are of a deutocerebral nature, unlike the similar structures in *Drosophila*. The present study differs from that of Sanchez in distinguishing between the tract from the β lobe to the antenno-motor centre, and the tracts from the cells of the glomerulus to the medial calyx (*A*, VIII, the olfactorio-globularis): the two tracts are not the same, as Sanchez suggests.

Several of the tracts listed above are shown to come from the suboesophageal region, but their origins here have not been traced: Kenyon has shown that some of the fibres from the β lobe make connexions with those from the thoracic ganglia. So little is known of the neurology of the suboesophageal ganglia that there is no other indication whether the fibres to the protocerebrum come from sensory or motor areas. The following considerations are however relevant here:

1. Tracts from the optic glomeruli and the antennal glomeruli, all of which are sensory centres, go only to the calyces and the α lobes.
2. Tracts from the β lobes go to the antenno-motor centre and the central body. The central body itself also receives tracts from all the sensory centres and is intimately connected with the suboesophageal ganglia.
3. The suboesophageal ganglia, apart from their role as centres for the mouthparts, have important functions in maintaining locomotor activity in the insect.

It therefore seems reasonable to suppose that the tracts from the suboesophageal ganglia to the calyces and α lobe are from sensory areas. It is further suggested that tracts from the β lobe are to motor regions; which implies that the central body has motor functions, although there is no physiological evidence on this point. These topics will be further discussed in the next section.

DISCUSSION

Since the mushroom bodies were first described, over a century ago, their function has been the subject of much speculation: all the fashionable mysteries of behaviour have been successively attributed to them. It is not proposed to add to these speculations here, but only to discuss the manner of functioning which their anatomical properties may confer upon them.

The descriptions given make it clear that the calyces and the α lobe both receive connexions from all the sensory centres of the brain. Although the

fibres entering the calyces and α lobe have not been counted, it is obvious that they are very numerous, and this, allied with the fact that a single afferent fibre makes synaptic connexions with many mushroom body fibres, makes it probable that each of the latter fibres has at least two connexions with sensory centres—one in the calyx and one in the α lobe. Although there is no evidence that the insect synapse is polarized, it seems more probable that the flow of

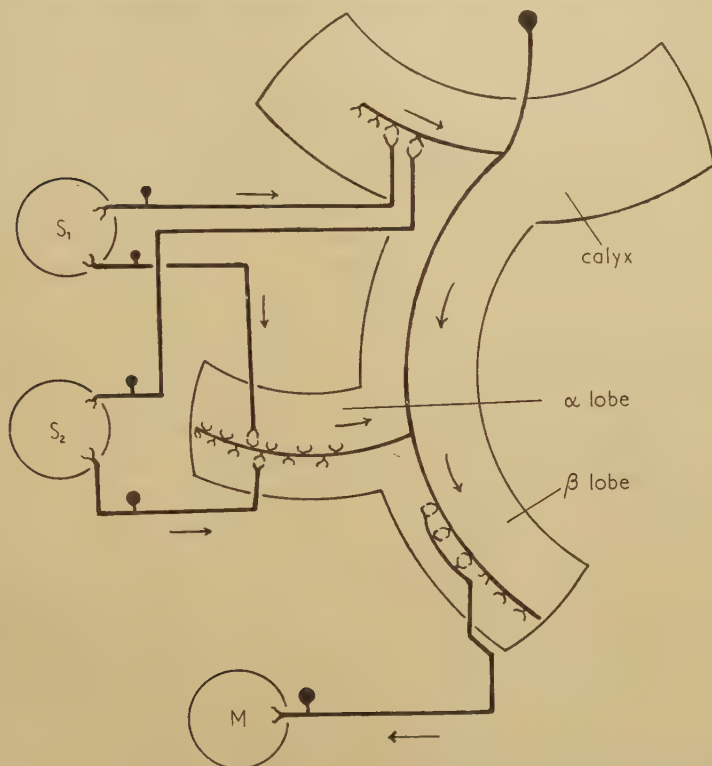


FIG. 9. Diagram to illustrate the suggested paths of conduction in the corpora pedunculata.

S_1 and S_2 = sensory centres.

M = motor centre.

excitation is from the sensory centres into the corpora pedunculata rather than the reverse. If this is so, then when the sensory centres are active each of the mushroom body fibres will be simultaneously excited both in the calyx and in the α lobe. If the two excitations are not synchronized, then the excitation which passes away down the β lobe must be the result of a combination or interaction of the two initial excitations in the calyx and α lobe. The possession of two reception areas, each with duplicate connexions, thus introduces the possibility of a type of comparator system which can be used to produce great variation in the transmitted information; this variation being caused by excitations arising in either the same or in different sensory centres. The mechanism for producing this variation will be discussed elsewhere.

The hypothesis given above is not the only one which would explain the facts. If, for example, it was supposed that tracts from the α lobe conducted excitation away from the lobe to the sensory centres, then some sort of self-exciting circuit might be set up, involving the series sensory centre \rightarrow calyx \rightarrow α lobe \rightarrow sensory centre \rightarrow &c., with a continual excitation passing away down the β lobe. Until some evidence is available to show that integrative centres may react upon sensory centres, it seems wiser, however, to assume that the flow of excitation normally passes from the sensory centres to the mushroom bodies, with the results already proposed.

If, as suggested, the β lobe is a transmitting region, then it is clear that tracts from this lobe must conduct excitation away to motor or integrative centres, as has already been suggested in discussing the origins of these tracts.

The polysynaptic connexions and large number of the fibres entering the calyx make it probable that the afferents must compete for the mushroom body fibres, which thus exemplify the 'convergence' and 'final common path' postulated for other neural organizations. If one afferent fibre can excite several post-synaptic fibres, then the calyx will act as a primitive amplifier. Perhaps more important, however, is the increased probability that in a population of post-synaptic fibres with varying thresholds at least a few will always be excited by the afferent impulse. This implies that on different occasions the same afferent fibre excites different post-synaptic fibres, which raises the question of the degree of localization of function, if any, which exists in the calyx. On anatomical grounds one might expect the different calyces, and the central and peripheral groups of cells within each calyx, to have different functions. The arrangement of the synaptic striae across the α lobe and along the β lobe, together with the observation that all the mushroom body fibres rigidly preserve their relative positions in these lobes, might indicate that the spatial distribution of the fibres has some functional significance. Any final determination of the functional localization must, however, depend upon physiological rather than histological observations.

It is indeed apparent that although we have a detailed knowledge of the histology of the corpora pedunculata, our ignorance of their function remains very great. Only physiological examination can clarify this matter, but it should be stressed that comparative studies of a physiological and a histological nature are complementary, and both are essential to a further understanding of the problem.

I wish to acknowledge the encouragement of Prof. K. D. Roeder at the beginning of this study, the valuable advice of Dr. T. C. Schneirla on tropical ants, the kindness of the Smithsonian Institution and their representative Dr. James Zetek in putting at my disposal the facilities of the Field Station on Barro Colorado Island, and the financial support of the Institute of International Education and the Royal Commissioners of the Exhibition of 1851. The cautery was suggested by Dr. R. H. J. Brown, devised by Mr. A. Popple, and partially constructed by Dr. W. Vanderkloot, to whom my thanks are due.

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A Comparative Morphological Study of the Tracheal System in Larval Diptera. Part I

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SUMMARY

The larval tracheal system of species representative of certain families in the Diptera Nematocera, Brachycera, and Cyclorrhapha is discussed. Detailed descriptions are given of the larval systems of members of the families Psychodidae, Trichoceridae, Anisopidae, Scatopsidae, Bibionidae, Phoridae, and Calliphoridae.

Attention is drawn to the fact that the dissimilarity between the tracheal systems of the species from the selected families—more particularly between the Diptera Nematocera and Cyclorrhapha—is superficial. The differences overlie what is in fact a series of tracheal systems in which the number and distribution of the main elements is remarkably constant. The homologies of these main elements in all three sub-orders are demonstrated. Correlation between the form of the central nervous system and the distribution of the tracheae is also shown.

Each described system is analysed into its main constituents and represented by a 'larval tracheal pattern'. On close examination the patterns or plans of each system in the various families are shown to conform to a 'general plan' common to all the species described, and probably representative of a general larval plan for the whole order; it is suggested that it approaches what must be the primitive distribution of tracheae in the Diptera. It is also suggested that this pattern may prove of phylogenetic importance should it be possible to discover similar generalized patterns for the other insect orders. It is shown how the plan can be used in interpreting previous descriptions by other workers.

The results presented illustrate the stability and remarkable constancy of the basic pattern of the tracheal system throughout the main sub-divisions of the order. No correlation is seen between the form of the internal tracheal system and the variations shown by the spiracles, the elements of the tracheal system remaining constant in number and origin in all the species described.

In a future paper it is proposed to describe the systems of species from the remaining nematoceran families; considerable deviation from this general plan is to be seen, but the systems can be interpreted in the light of the present results.

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INTRODUCTION

MANY workers have described the larval tracheal system of individual dipteran species, but no attempt has been made to present a comparative morphological account. In fact few comparative studies on this system have been made for any of the insect orders. Notable exceptions are those of Fuller for the Isoptera (1919), Landa for the Ephemeroptera (1948), and the work of Lehmann (1925) who surveyed the tracheal system of the whole class. The earlier classical work of Palmén (1877) provides the basic work on topography and anatomy of the insect tracheal system, and many of Lehmann's deductions are based on this.

The detail with which the tracheal systems of individual dipteran species have been described varies considerably. Very many accounts, both older and more recent, are very incomplete, while many of the more detailed accounts throw no light on the homologies of the individual parts. In fact, the systems vary so considerably within the order that it would appear that no consistency exists, and it is therefore not surprising that comparisons have been made only infrequently.

Older accounts, such as those of Lowne (1900) for *Calliphora erythrocephala* (Meigen), Hewitt (1914) for *Musca domestica* Linnaeus, and de Meijere (1901) for *Lonchoptera* sp., are often vague and incomplete. Ruhle's (1932) description of *Drosophila melanogaster* Meigen—which is quoted by Bodenstein (in Demerec, 1950)—and also Stammer's (1924) for *Tabanus* sp., give a good general account, but many details are not included, while Kemper's (1925) description for *Psychoda phalaenoides* (Linnaeus), and Fuerborn's (1927) description of *Psychoda sexpunctata* Curtis are detailed. Lehmann's (1925) survey of the insect tracheal system is based on a number of the older descriptions including the work of Palmén and those of Wahl (1900) for *Eristalis tenax* (Linnaeus), Taylor (1902) for *Simulium latipes* (Meigen), and Hewitt for *Musca domestica* (1914); these vary considerably in their degree of detail. Wardle (1926) describes in detail, and compares, the systems of two tipulids, *Tipula flavolineata* Meigen and *Pedicia rivosa* (L.). However, he does not attempt to homologize the individual tracheae. The account by Gäbler (1930) of the tracheal system of *Eristalis tenax* is very detailed, clear, and instructive. Cameron (1933), after describing the system of *Haematopota pluvialis* (Linnaeus), proceeds to compare it with Stammer's (1924) description for *Tabanus* sp. However, the anterior region is such a complicated mass of tracheae in both, that homologizing of all the parts has obviously presented difficulties. These difficulties would have been far less were the homologies of the main tracheae known.

The only recent comparative work on any aspect of the tracheal system appears to be that of Keilin (1944). He summarizes past work on the detail of the spiracles, and describes the metamerism and the process of moulting of the tracheal system in larval Diptera. He is not concerned with the detailed morphology of the individual tracheae nor with their homologies. Hence his

description of the dipteran larval system is not based on a comparative study of the detailed morphology of the individual elements.

Larvae of some thirty identified species have been studied in detail during the present investigation. The species have, as far as was possible, and as time permitted, been taken from different sub-divisions of the Diptera. The results obtained from these and other species studied, together with evidence supplied from descriptions by other workers (which as the above résumé briefly indicates, is in some cases detailed, in others fragmentary), have been pieced together and an attempt is made to give a more complete picture of the larval tracheal system in the Diptera, than has previously existed.

In the present paper descriptions are given of a number of specially selected dipteran larvae whose tracheal systems most closely approach what the results of the investigation indicate to be the generalized larval dipteran tracheal system. In a future paper descriptions will be given of the more anomalous systems found in the remaining larval Diptera which have been studied.

Species described and figured

Nematocera

Family Psychodidae: *Psychoda alternata* Say

Family Trichoceridae: *Trichocera annulata* Meigen

Family Anisopidae: *Anisopus fenestralis* (Scopoli)

Family Bibionidae: *Dilophus febrilis* (Linnaeus)

Family Scatopsidae: *Scatopse notata* (Linnaeus)

Brachycera and Cyclorrhapha

Family Phoridae: *Aphiochaeta (Megaselia)* sp.

Family Calliphoridae: *Calliphora erythrocephala* (Meigen)

MATERIALS AND METHODS

Living larvae were obtained by either collecting or breeding through from eggs laid by females in captivity. Specimens of *Psychoda alternata* were obtained from the sewage beds at Cambridge; *Trichocera annulata* was obtained from the same habitat; *Anisopus fenestralis* was bred through from eggs kindly sent by Dr. Ll. Lloyd of Leeds, and others were obtained from cow-dung and rotting fruit and from eggs laid by females in captivity; *Dilophus febrilis* was bred right through from larvae found in cow-dung; *Scatopse notata* was bred from eggs laid by females which had emerged from fungi collected and kept in the laboratory; *Aphiochaeta (Megaselia)* sp. in all its stages was obtained from decaying recently killed cockroaches belonging to a laboratory culture.

Each was studied in the living state, either in a drop of water between slide and coverslip, or by dissection in glycerine. In a few cases, permanent preparations were made using an injecting technique (Wigglesworth, 1950). This

involves the removal of air from the tracheal system of a living larva by means of a vacuum pump, followed by immersion of the larva in cobalt naphthalate dissolved in white spirit. Subsequent rinsing in white spirit removes cobalt naphthalate from the surface of the larva. Hydrogen sulphide bubbled through white spirit containing the specimen precipitates the cobalt in the tracheae as cobalt sulphide. The larva is then fixed, cleared and mounted in canada balsam.

Illustrations

In most cases the larva is drawn from the dorsal side; where detail is obscure a portion is drawn on a larger scale. Opposite each figure is a diagrammatic representation of the particular system to show the distribution of the elements and their relation to the spiracles together with the functional or non-functional nature of these. In each plan the right half of the larval system only is given: the centre of the diagram represents the right lateral view, the left the right dorsal side, and the right the right ventral elements of the system. The diagrammatic representations enable easier comparison between one system and another, as the main elements are frequently obscured by fine tracheae, or their distribution is masked in some other way. In all cases the figures omit the finest tracheae and tracheoles.

The descriptive terms 'dorsal' and 'lateral longitudinal trunks', 'transverse connectives', 'spiracular', and 'visceral tracheae', are those used by previous workers. However, the terms 'dorsal' and 'ventral cervical tracheae', 'dorsal cervical anastomosis', 'ventral ganglionic tracheae', and 'ventral thoracic anastomoses' have not, to the writer's knowledge, been consistently used before; they are those found to be, descriptively, the most appropriate.

According to Keilin (1944) the spiracles are intersegmental in origin. This is explained by the forward migration of the so-called 'pro-thoracic' spiracles, and the backward migration of all the other pairs of spiracles. The present work shows that the internal morphology of the system substantiates this view. Hence in the following descriptions the terms 'pro-thoracic' and 'meta-thoracic' spiracles (which imply the absence of a meso-thoracic spiracle) will be replaced by 'anterior' and 'posterior' thoracic or 'first' and 'second thoracic' pairs of spiracles.

THE GENERALIZED DIPTERAN LARVAL SYSTEM

As mentioned previously, the only attempt at describing a generalized dipteran larval system is that by Keilin (1944). He takes *Scatopse notata* as a typical example and enumerates the following components.

(1) two main longitudinal latero-dorsal trunks; (2) a pair of secondary latero-ventral longitudinal trunks; (3) transverse branches which are given off laterally from the main tracheal trunks and connect them, in each segment, with the latero-ventral trunks; they continue as spiracular branches terminating in more or less developed spiracles; (4) transverse commissures which connect, in each segment, the main tracheal trunks; (5) tracheal branches or tufts, which arise principally from

the secondary latero-ventral trunks, the ramifications of which extend to various organs; (6) branches which arise from transverse commissures; (7) spiracles, functional or non-functional, and perispiracular glands.

In each of the species to be described in this paper the following parts can be distinguished (fig. 1):

(1) Ten spiracles (sp.).

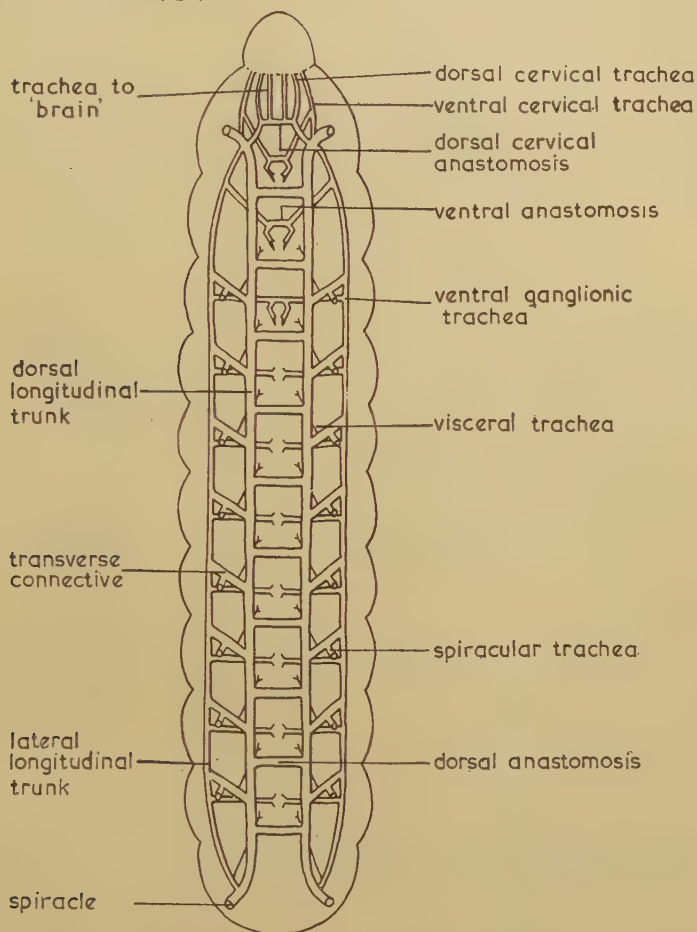


FIG. 1. Dorsal view of the generalized larval tracheal system.

- (2) Two dorsal longitudinal trunks (d.l.t.).
- (3) Two lateral longitudinal trunks (l.l.t.).
- (4) Eight transverse connectives (tr.c.).
- (5) Ten dorsal anastomoses (d.a.).
- (6) Two dorsal cervical tracheae (d.c.).
- (7) One dorsal cervical anastomosis (d.c.a.).
- (8) Two ventral cervical tracheae (v.c.).

- (9) Ventral ganglionic tracheae (v.g.).
 - (a) Three thoracic, with ventral anastomoses (v.a.).
 - (b) Seven abdominal, without ventral anastomoses.
- (10) Visceral tracheae (vcl.).

It can be seen that components (1), (2), (3), (4), and (10) are those mentioned by Keilin although the exact number of components is not given by him. Numbers (6), (7), (8), and (9) are not mentioned or figured in his paper. These latter are, moreover, the elements most frequently omitted in descriptions by other workers, but are definitely represented in all the Diptera observed by the present author. In taking *Scatopse notata* as his example Keilin specified eleven as the normal number of dorsal anastomoses, whereas it will be seen that from the present results the normal number appears to be ten.

THE TRACHEAL SYSTEM OF INDIVIDUAL SPECIES

1. *The tracheal system in Psychodidae*

Psychoda alternata

The larva has a head capsule (in common with most Nematocera). The system figured is that of a fourth instar larva; it is amphipneustic, having the post-abdominal spiracles opening at the end of a respiratory siphon. The metameric nature of the system is clearly seen and the whole can be analysed into the following components (fig. 2):

Dorsal longitudinal trunks (d.l.t.). There are two of these extending from the first pair of thoracic spiracles to the last pair of abdominal spiracles.

Lateral longitudinal trunks (l.l.t.). These are also paired and run the whole length of the larva.

Transverse connectives (tr. c.). In addition to their anterior and posterior junctions, the dorsal and lateral longitudinal trunks of either side are linked by eight transverse connectives. It is from these that the spiracular threads arise.

Dorsal anastomoses (d.a.). The dorsal longitudinal trunks are connected by ten commissures, the first and second arising between the region of the first thoracic spiracles and first transverse connectives, and one between each of the subsequent connectives and the post abdominal spiracles.

Dorsal cervical tracheae (d.c.). On each side, a trachea arises in the region of the first thoracic spiracle, and appears almost as a continuation of the dorsal longitudinal trunk. Each passes anteriorly to the head after giving rise to two branches. The posterior one divides, one part passing to the supra-oesophageal ganglia (s. oes.) and the other to the sub-oesophageal ganglion (sb. oes.) which lie outside the head capsule. The second pair are given off medially and anastomose mid-dorsally (d.c.a.) just posterior to the head capsule.

Ventral cervical tracheae (v.c.). These also occur in the region of the first thoracic spiracle and appear as continuations of the lateral longitudinal trunks. They also pass anteriorly into the head capsule.

Ventral ganglionic tracheae (v.g.). These pass to the nerve ganglia comprising

the ventral nerve chain. The first pair is given off by the ventral cervical tracheae, and the subsequent ones by the segments of the lateral longitudinal trunk of either side. Each passes ventrally to the nerve ganglion of its segment. The first three constitute the thoracic tracheae, and are peculiar in forming

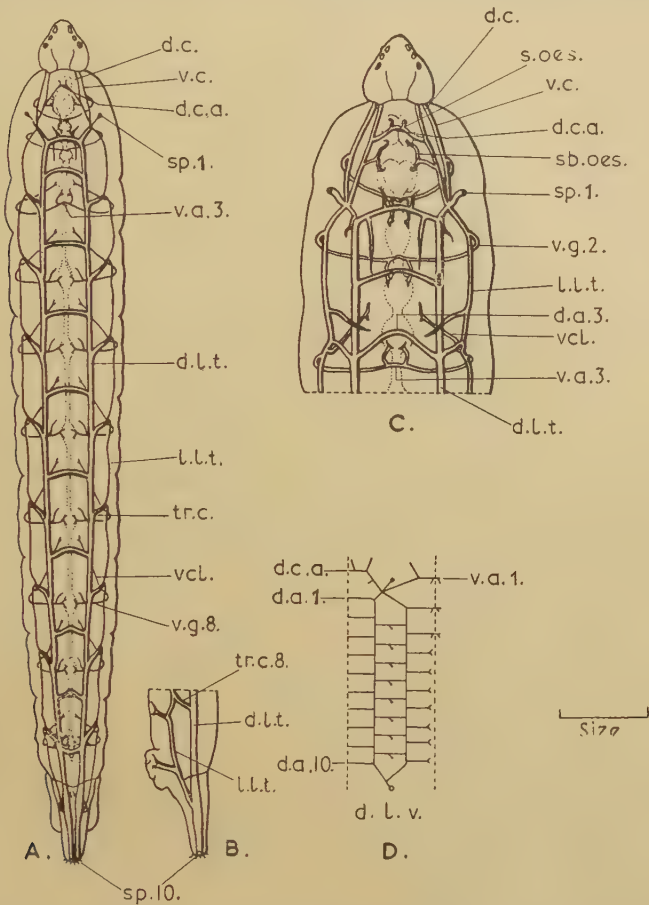


FIG. 2. The larval tracheal system of *Psychoda alternata*. A, dorsal view of the larval system. B, posterior end in lateral view. C, anterior end in dorsal view. D, plan of the right half of the larval system.

mid-ventral anastomoses (v.a.); the corresponding abdominal tracheae do not anastomose mid-ventrally.

Visceral tracheae (vcl.). On both sides, and in each segment, visceral tracheae arise just anterior to the origin of the ventral ganglionic tracheae.

2. Family Trichoceridae

Trichocera annulata

The following description is of a fourth instar larva which has a head capsule and is amphipneustic; the last pair of spiracles opens at the posterior end of the

body and is surrounded by four lobes. The tracheal system consists of the following parts (fig. 3):

Dorsal and lateral longitudinal trunks (d.l.t., l.l.t.). Both pairs of trunks are present and are well developed.

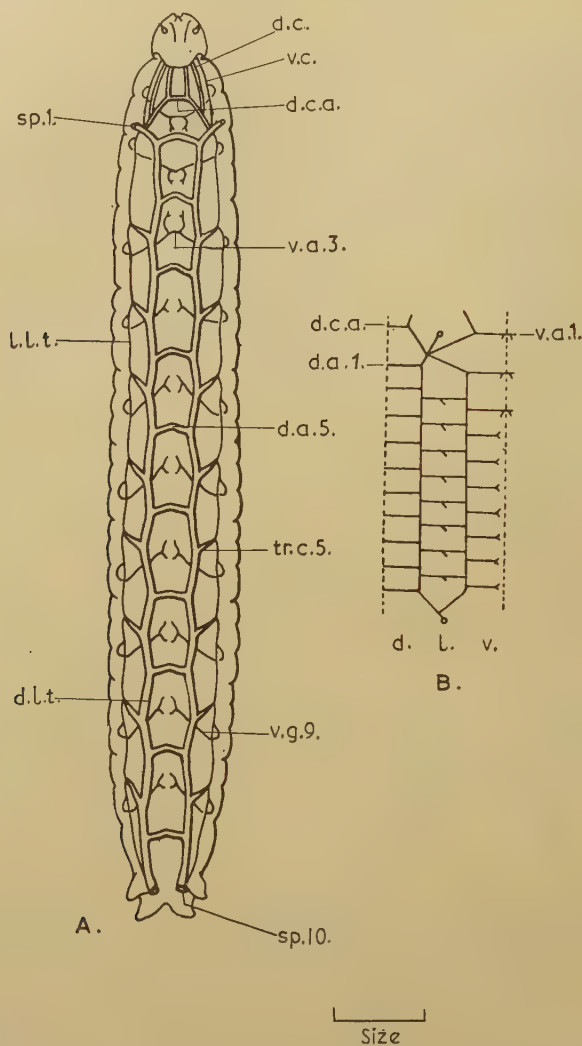


FIG. 3. The larval tracheal system of *Trichocera annulata*. A, the larval system in dorsal view. B, plan of the right half of the larval system.

Dorsal anastomoses (d.a.). The two longitudinal trunks are connected dorsally by ten commissures, the first two occurring in the region between the functional first thoracic spiracles and first transverse connectives (t.c.), while the remainder occur between each subsequent pair of connectives and the last pair of spiracles.

Transverse connectives (t.c.). Eight of these are present and unite the dorsal and lateral trunks of either side.

Dorsal cervical tracheae (d.c.). These arise just anterior to the junction of the longitudinal trunks. Before entering the head, a median branch is given off which anastomoses mid-dorsally with its fellow to form the dorsal cervical anastomosis (d.c.a.).

Ventral cervical tracheae (v.c.). These also arise in the region of the first thoracic spiracle and pass into the head.

Ventral ganglionic tracheae (v.g.). A pair of tracheae serves each nerve ganglion. The first pair arises from the ventral cervical tracheae, and the remainder from the lateral longitudinal trunks. The thoracic tracheae form three mid-ventral anastomoses (v.a.).

As only a limited number of larvae were available the system has not been studied in as great detail as the other species described. No descriptions of the tracheal system of any of the Trichoceridae have been seen in the literature.

3. Family Anisopidae

Anisopus fenestralis

The larva has a head capsule; the following description is of a fourth instar larva which is amphipneustic, having well developed first thoracic and terminal abdominal spiracles. The tracheal system is composed of the following parts (fig. 4):

Dorsal and lateral longitudinal trunks (d.l.t., l.l.t.). These two pairs of trunks are present and well developed.

Transverse connectives (t.c.). Besides their union anteriorly and posteriorly, the dorsal and lateral trunks of either side are joined by eight transverse connectives.

Dorsal anastomoses (d.a.). The dorsal trunks are connected by ten dorsal anastomoses, one occurring between each pair of transverse connectives, the tenth between the last connective and the last pair of abdominal spiracles, while the first and second occur between the first thoracic spiracles and first transverse connectives.

Dorsal cervical tracheae (d.c.). These arise near the junction of the longitudinal trunks with the first thoracic spiracular tracheae, and pass anteriorly into the head capsule. A median branch is given off from each and this subdivides; one part anastomoses mid-dorsally with its fellow (d.c.a.) and the second runs, first in a posterior direction and then anteriorly to what are the supra-oesophageal ganglia lying outside the head capsule.

Ventral cervical tracheae (v.c.). These also run anteriorly into the head capsule, first giving off ventral tracheae to the pro-thoracic ganglion.

Ventral ganglionic tracheae (v.g.). Each ganglion is served by a pair of tracheae; the first pair arises from the ventral cervical tracheae, and the remaining pairs from the lateral trunk. Those passing to the three thoracic ganglia form mid-ventral anastomoses (v.a.).

Visceral tracheae (vcl.). These arise from the lateral trunk just anterior to its junction with each of the transverse connectives.

The tracheal system of *Anisopus* does not appear to have been described previously.

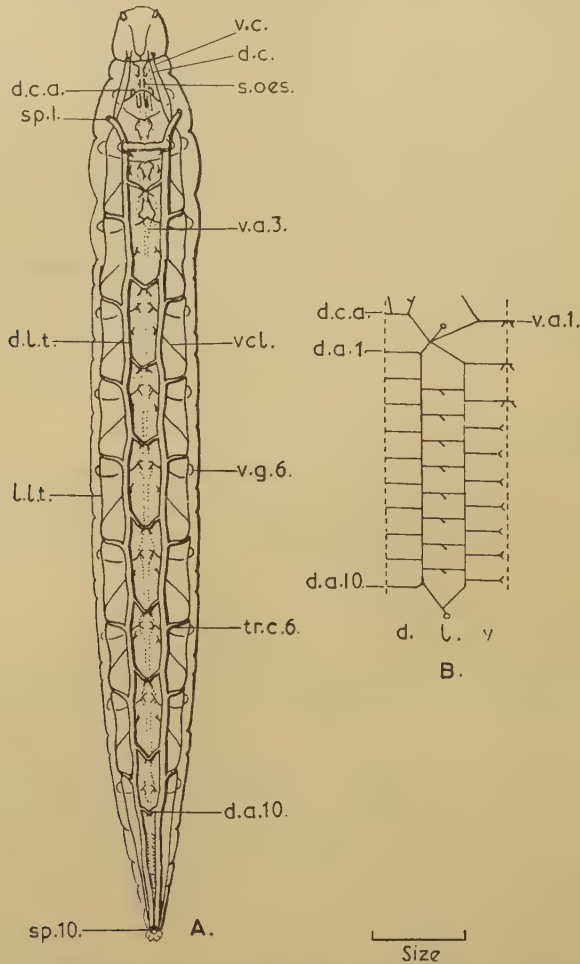


FIG. 4. The larval tracheal system of *Anisopus fenestralis*. A, the larval system in dorsal view. B, plan of the right half of the larval system.

4. Family Bibionidae

Dilophus febrilis

The larva has a head capsule. The following description is of the fourth instar larva which is holopneustic; the first thoracic spiracles are on the second apparent segment and the second pair on the meta-thoracic segment. Of the abdominal pairs, the last is very large and is situated at the anterior margin of

the last segment. The tracheal system is composed of the following parts (fig. 5):

Dorsal and lateral longitudinal trunks (d.l.t., l.l.t.). There are two well-developed pairs present.

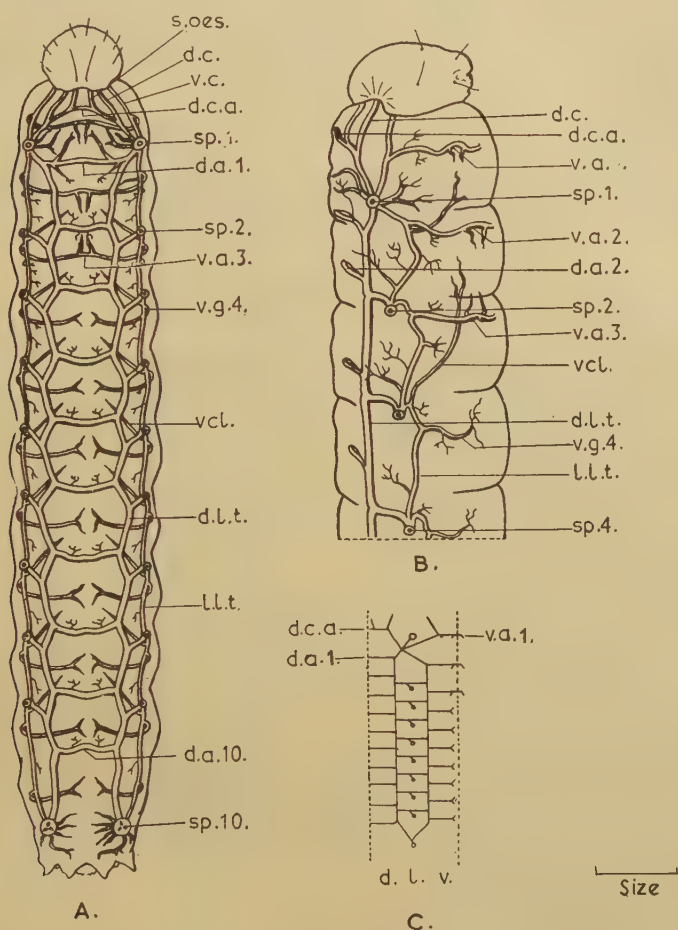


FIG. 5. The larval tracheal system of *Dilophus febrilis*. A, dorsal view of the larval system. B, the anterior end in lateral view. C, plan of the right half of the larval system.

Transverse connectives (t.c.). The two trunks of either side are connected by eight tracheae, each of which gives rise to a spiracular branch.

Dorsal anastomoses (d.a.). These trunks are connected by ten dorsal anastomoses, two occurring between the first and second thoracic spiracles and the remainder between each of the subsequent pairs of spiracles.

Dorsal cervical tracheae (d.c.). These originate near the junction of the dorsal and lateral trunks, and pass into the head capsule. Before doing so, a median branch is given off which divides; one part anastomoses mid-dorsally with its

fellow (d.c.a.), and the second passes into the head alongside the main trachea (s. oes.).

Ventral cervical tracheae (v.c.). These also pass into the head capsule.

Visceral ganglionic tracheae (v.g.). The first pair of ventral tracheae arises from the ventral cervical tracheae and passes to the first thoracic ganglion. The subsequent ventral tracheae arise from the lateral trunk. Those passing to the three thoracic ganglia form mid-ventral anastomoses (v.a.), whereas the abdominals do not.

Visceral tracheae (vcl.). These arise from the spiracular region of segments two to nine inclusive.

In the region of the first thoracic spiracles there are many other tracheae, which are figured but will not be described in detail.

5. Family Scatopsidae

Scatopse notata

The larva has a head capsule. The following description is of the fourth instar larva which is peripneustic. The last pair of spiracles is situated on slight protuberances of the last abdominal segment. The tracheal system consists of the following parts (fig. 6):

Dorsal and lateral longitudinal trunks (d.l.t., l.l.t.). Both pairs are present and extend throughout the length of the larva.

Dorsal anastomoses (d.a.). The dorsal trunks are connected by ten dorsal anastomoses. The first two occur in the region between the first thoracic spiracles and the non-functional second thoracic spiracles; the third to ninth occur in the region between successive abdominal spiracles, and the tenth and eleventh between the region of the ninth and tenth pair of spiracles.

Transverse connectives (t.c.). Eight of these join the dorsal and lateral longitudinal trunks on each side.

Dorsal cervical tracheae (d.c.). These arise at the junction of the first thoracic spiracular tracheae with the longitudinal trunks, and pass forward to enter the head. Before entering, a median trachea is given off which divides, one part passing alongside the main trachea (s. oes.) and the second anastomosing with its fellow mid-dorsally (d.c.a.).

Ventral ganglionic tracheae (v.g.). One pair of tracheae passes to each of the nerve ganglia. The first pair arises from the ventral cervical tracheae, and the remainder from the lateral longitudinal trunks. The three thoracic pairs form mid-ventral anastomoses (v.a.), whereas the abdominal ones do not.

Keilin (1944) figures the larval system of *Scatopse notata*, but omits the ventral cervical tracheae and all ventral ganglionic tracheae, including the three mid-ventral anastomoses.

6. Family Phoridae

Aphiochaeta (*Megaselia*) sp.

The larval head is non-capsular, being reduced to mouth hooks and sup-

porting skeleton. The following description is of a third instar larva which is amphipneustic; the first thoracic spiracles are distinct, and the abdominal ones terminal. The tracheal system consists of the following parts (fig. 7):

Dorsal and lateral trunks (d.l.t., l.l.t.). These are present and well developed.

Transverse connectives (t.c.). In addition to their anterior and posterior union the dorsal and lateral trunks are joined by eight transverse connectives.

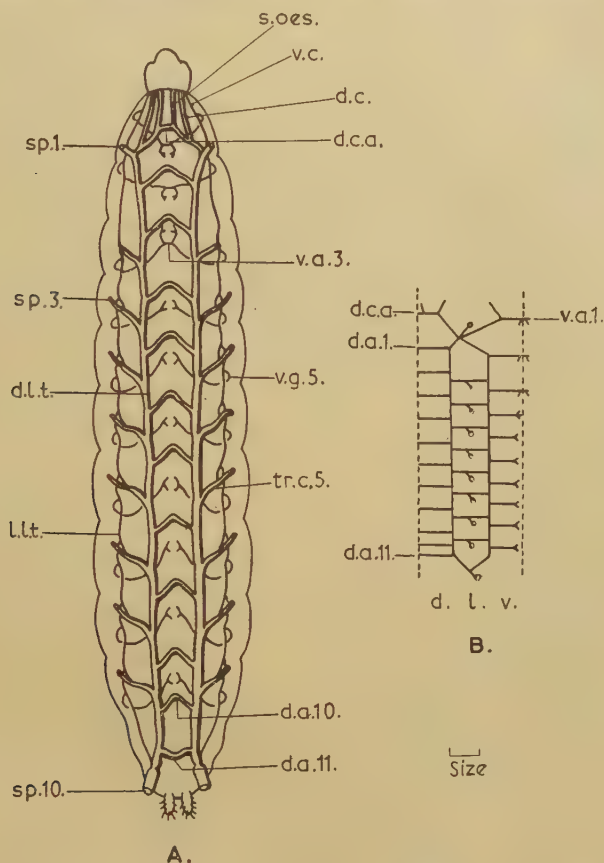


FIG. 6. The larval system of *Scatopse notata*. A, the larval system in dorsal view. B, plan of the right half of the larval system.

Dorsal anastomoses (d.a.). The dorsal trunks are connected by ten dorsal anastomoses. The first two occur between the first thoracic spiracles and the first transverse connectives, the second to ninth between the successive transverse connectives, and the tenth, which is much shorter and stouter, between the last connectives and last pair of spiracles.

Dorsal cervical tracheae (d.c.). These arise just posterior to the junction of the lateral and dorsal longitudinal trunks. The main trachea passes anteriorly, but shortly after its origin it gives rise to a trachea which passes posteriorly to unite with its fellow and form a deep median anastomosis (d.c.a.). From the

latter two tracheae pass to the posteriorly displaced supra-oesophageal ganglia (s. oes.).

Ventral cervical tracheae (v.c.). These arise slightly anterior to the dorsal

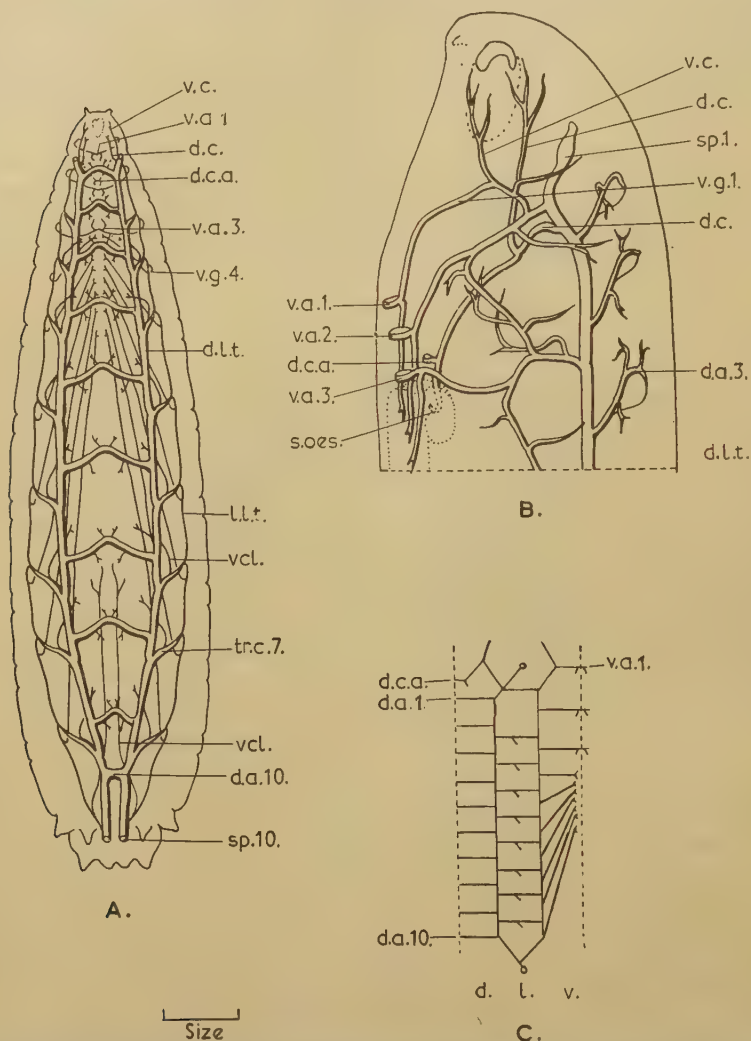


FIG. 7. The larval tracheal system of *Aphiochaeta (Megaselia)* sp. A, the larval system in dorsal view. B, the anterior end in left lateral view. C, plan of the right half of the tracheal system.

cervicals. Each first gives rise to a superficial dorsal branch, then to the first ganglionic tracheae and finally passes anteriorly.

Ventral ganglionic tracheae (v.g.). As the nervous system is concentrated in the thoracic region, all the pairs are correspondingly displaced. The first three, which constitute the thoracic tracheae, form mid-ventral anastomoses (v.a.).

Visceral tracheae (vcl.). These arise from the transverse connectives in every

segment, but that arising from the lateral trunk before its posterior junction with the dorsal trunk is very much larger than the others.

As far as is known to the writer there is no existing description of the tracheal system of any of the Phoridae.

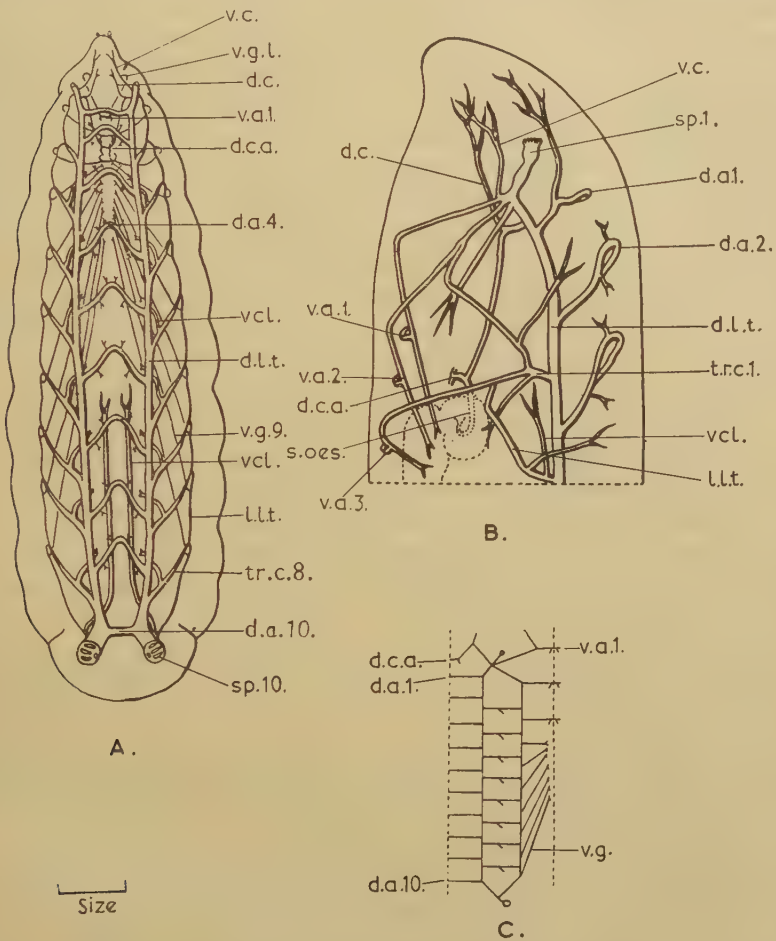


FIG. 8. The larval tracheal system of *Calliphora erythrocephala*. A, dorsal view of the larval system. B, the anterior end in left lateral view. C, plan of the right half of the system.

7. Family Calliphoridae

Calliphora erythrocephala

In common with other Cyclorrhapha the larval head is non-capsular, being reduced to mouth hooks and supporting skeletal elements. The third instar larva is amphipneustic, with first thoracic, and very large terminal abdominal spiracles. The tracheal system is composed of the following elements (fig. 8):

Dorsal and lateral longitudinal trunks (d.l.t., l.l.t.). Two well-developed pairs are present,

Transverse connectives (t.c.). Besides their junction anteriorly and posteriorly the dorsal and lateral trunks are also joined by eight transverse connectives.

Dorsal anastomoses (d.a.). The dorsal trunks are connected dorsally by ten dorsal anastomoses. The first and second lie between the first thoracic spiracles and the first transverse connectives, numbers three to nine between successive transverse connectives, and number ten between the eighth connective and last pairs of abdominal spiracles. The first anastomosis is wider than numbers two to nine while the tenth is very much stouter and shorter than the rest.

Dorsal cervical tracheae (d.c.). These arise just posterior to the junction of the dorsal and lateral trunks. The main trachea passes anteriorly, but shortly after its origin it gives off a trachea which passes posteriorly to unite with its fellow and form a deep median anastomosis (d.c.a.). From this, two tracheae pass to the posteriorly displaced supra-oesophageal ganglia (s. oes.).

Ventral cervical tracheae (v.c.). These arise anterior to the dorsal cervicals and pass anteriorly. A dorsal superficial trachea is first given off and then the first ganglionic.

Ventral ganglionic tracheae (v.a.). The first pair of these originates from the ventral cervical tracheae and subsequent ones from the lateral trunks. As the nervous system is concentrated in the thoracic region, the tracheae are correspondingly displaced. The three anterior thoracic pairs form mid-ventral anastomoses (v.a.), whereas the abdominals do not.

Visceral tracheae (vcl.). These spring from the transverse connectives in each segment. The trachea arising from the lateral trunk shortly before its junction posteriorly with the dorsal trunk is very much larger than are any of the others.

Although the 'typically cyclorrhaphan' larval tracheal system is a familiar one, accurate descriptions are virtually non-existent, Gäbler's (1930) being a noteworthy exception.

Larvae of undetermined species of *Musca*, *Fannia*, and *Cordilura* (observed by the writer), all of which are calypterates, have tracheal systems very similar to that of *Calliphora*. This similarity extends to the acalypterates. Ruhle's (1932) description of the development of the tracheal system in the larva of *Drosophila melanogaster* has been confirmed during the present investigation, and the system is seen to agree very closely with those of the Calyptratae. The larva of *Eristalis tenax* (Cyclorrhapha, Aschiza) is described by Gäbler (1930), and his detailed account of the system is also very similar to all of the above, except for the incomplete connexion of dorsal anastomoses two and three in *Eristalis*. Imms' (1942) diagram of the tracheal system of the larva of *Braula coeca* is also 'typically Cyclorrhaphan' except that only nine dorsal anastomoses and seven transverse connectives are figured. This suggests the absence of a complete tracheal segment, which may possibly have been overlooked.

DISCUSSION

It has been seen that the tracheal systems of the above larvae possess a remarkably similar arrangement of elements, giving almost identical larval patterns. From these the generalized larval pattern has been deduced.

The variations seen in the spiracles—which vary from the amphipneustic condition found in Psychodidae, Trichoceridae, Anisopidae, Phoridae, and Calliphoridae, through the peripneustic condition of the Scatopsidae to the holopneustic condition in the Bibionidae—are not accompanied by any corresponding modifications of the internal morphology of the tracheal system.

The *ten dorsal anastomoses* are seen to occupy a definite position. Numbers one and two arise from the dorsal longitudinal trunk between the first and second pairs of spiracles. The remainder arise from the trunks between each of the consecutive pairs of spiracles (second to tenth). In *Scatopse notata* an eleventh anastomosis occurs between the tenth and last pair of abdominal spiracles; this is more probably an addition to the normal complement of ten; if it were not so and the primitive number were eleven then the condition of all the other described species would be secondarily derived by the loss of the eleventh anastomosis.

Most descriptions by other workers usually include the longitudinal trunks, transverse connectives, and at least some of the dorsal anastomoses. In de Meijere's description of *Lonchoptera* (1901) the relationship of these tracheal elements at the posterior end of the larva is clear, but at the anterior end they are very incompletely figured. Nine tracheal metameres, and not ten, are figured by Imms for *Braula coeca* (1942); that is, there are present only nine dorsal anastomoses instead of the usual ten and only seven transverse connectives instead of the normal number of eight. There would be correspondingly nine spiracles and not ten if these were functional and figured. Either this is a descriptive error or the system is remarkably peculiar in this respect. Similarly only seven transverse connectives are described for *Lonchoptera* sp. (de Meijere, 1901).

In the region anterior to the first thoracic spiracles two pairs of tracheae are seen to arise in each of the described species. Both pairs pass into the head in forms having an obvious head capsule, and in the case of those in which the head is non-capsular but reduced to mouth hooks, these elements can be recognized by the distribution of the tracheae arising from them. The ventral pair—the *ventral cervical tracheae*—in all cases give rise to tracheae which pass to the pro-thoracic ganglion. Without exception, the dorsal pair—the *dorsal cervical tracheae*—give off median branches which join mid-dorsally in the characteristic *mid-dorsal cervical anastomosis*. From the dorsal anastomosis there arise two tracheae which pass to the 'brain', composed of the supra-oesophageal ganglia and sub-oesophageal ganglion. This anastomosis and the cervical tracheae are not mentioned or are incompletely figured for *Calliphora* (Lowne, 1900), *Musca* (Hewitt, 1914), *Metoponia*—Stratiomyidae—(Irwin-Smith, 1923), *Haematopota* (Cameron, 1933), and *Tabanus* (Stammer, 1924), although they are almost certainly present in each of these genera.

In all of the described species the *ventral ganglionic tracheae* tracheate the thoracic and abdominal elements of the central nervous system. Typically, they arise from the lateral longitudinal trunks and each has a characteristic

position of origin. The tracheae to the pro-thoracic ganglion differ from the remainder of the ganglionic tracheae in arising from the ventral cervical tracheae; those passing to the meso-thoracic ganglion arise from the lateral trunks half-way between the first and second pairs of spiracles; those to the meta-thoracic and all abdominal ganglia arise from the lateral trunks in the region between consecutive pairs of spiracles. A remarkable feature is that the tracheae passing to the thoracic ganglia give rise to *mid-ventral anastomoses*. These are recorded in very few accounts given by other workers, although they have been observed in all the species described in this paper, and in numerous other species investigated by the writer; not one cyclorrhaphan or brachyceran has been found in which they are absent. The ganglionic tracheae are imperfectly represented and the ventral anastomoses are not described in most accounts, including those of Hewitt (1914), Imms (1942), de Meijere (1901), Ruhle (1932), Cameron (1933), Stammer (1924), and Irwin-Smith (1923). They are probably present though unobserved or unmentioned in the species described by these authors. The present condition in which the thoracic tracheae only form ventral anastomoses is possibly derived from an ancestral condition in which abdominal ganglionic tracheae also possess them.

The *dorsal cervical anastomosis* and the *ventral ganglionic tracheae* are constant in their origin and in the distribution of their tracheae. They do, however, vary considerably in their position. At first sight their position in the Cyclorrhapha would appear to be characteristic and diagnostic of this sub-order, being very different from the condition in the majority of Nematocera. However, the systems of the species described above show that the position is governed solely by the form of the central nervous system, which in the nematoceran species described here is in the form of distinct ganglia extending the length of the larva, but in the Cyclorrhapha is concentrated into a mass lying in the post-thoracic and anterior abdominal region. The present writer has observed both in certain of the brachyceran families such as the Leptidae—where the central nervous system may attain a nematoceran form—and in others, such as Tabanidae and Stratiomyidae—where it is concentrated—that the ganglia remain distinct and a single pair of ventral ganglionic tracheae tracheate each ganglion. In the Cyclorrhapha in which external evidence of segmentation in the nerve mass has disappeared, the segmentally arranged ganglionic tracheae are the only remaining external indication of the fundamentally segmented nature of the nervous system. The 'brain' can be seen to lie inside the head capsule in Trichocera, Scatopse, and Dilophus, just outside in Psychoda and Anisopus, and situated in the posterior thoracic region in Aphiochaeta, Calliphora, and all Cyclorrhapha; in general the position of the dorsal cervical anastomosis is correspondingly displaced.

It seems probable that the generalized tracheal pattern given at the beginning of this paper approaches that of the ancestral larval dipteran pattern, and it should thus possibly prove of interest in the phylogenetic relationships of the order, especially if a similar type of pattern should be discovered for other

insect orders. Improvement on Lehmann's (1925) deductions on the form of the insect tracheal system could follow only after each order of insects is thoroughly investigated. Only by first tracing the evolution of the tracheal system within each order can any fruitful attempt be made at tracing the relationships of the system within the class as a whole.

The fact that the main tracheal elements are identical in number and origin in certain nematoceran families, in the Brachycera seen to date, and in the Cyclorrhapha, is remarkable and is a positive reply to the question of the stability of the system, for the tracheal pattern composed of the *main* elements has remained unchanged in spite of the wide diversity of larval habits, ranging from purely terrestrial forms to wholly aquatic types. Furthermore, this consistency is maintained while, among the same species, considerable variation occurs in the condition of the spiracles.

The tracheal system is therefore seen to have remained remarkably unaltered in an order in which considerable variation has occurred in other characters. The larval head capsule and the male genitalia (Crampton, 1942), both external characters, have been used as systematic characters in tracing phylogenetic relationships within the Diptera. It is therefore conceivable that where variations in the tracheal systems are found to occur this system may also prove to be a useful systematic character. The tracheal system would appear to have a certain advantage over these other characters in being internal, and therefore less influenced by external conditions.

One example of the stability of the tracheal system may be illustrated by the 'rat tailed' larva of *Eristalis tenax*. The larva is strikingly adapted to live in water several inches deep, breathing by means of its last pair of spiracles which are situated at the extreme tip of a very long retractile syphon. If the tracheal system is unstable it might be expected that such a remarkably adapted larva, whose relatives are terrestrial, would have a correspondingly highly modified internal tracheal system. On the other hand, the same organs have to be supplied with tracheae in aquatic and terrestrial forms. Details taken from Gäbler's (1930) description of the larval system of this species show that, in actual fact, no great modification has taken place, and the main tracheae are identical with those of the general tracheal pattern given here for the whole of the order. Critical examination of the figures given by Gäbler makes it clear that the only modification lies in the enormous elongation of the tenth spiracular tracheae.

It is hoped that the general tracheal pattern which has been deduced will prove valuable in further studies and also in examining the descriptions of other workers. An example of the possibilities in this respect can be illustrated by taking Cameron's (1933) work on *Haematopota pluvialis*. He described in some detail the larval tracheal system of this species although no mention is made of the branches tracheating the nervous system. He compares the system with that of *Tabanus* species, described by Stammer (1924). The anterior ends of both larvae contain numerous tracheae whose relationship is described by him as being difficult to interpret. The figures and lettering of

Cameron and Stammer are reproduced here (fig. 9). Cameron concludes his comparison thus:

'... the distribution of the non-segmental branches 1-6, 8 and 9 at the anterior end of the longitudinal trunk is not easily traceable. . . . identity of the branches has been established in all save two instances—7 and 9, and there are differences regarding the

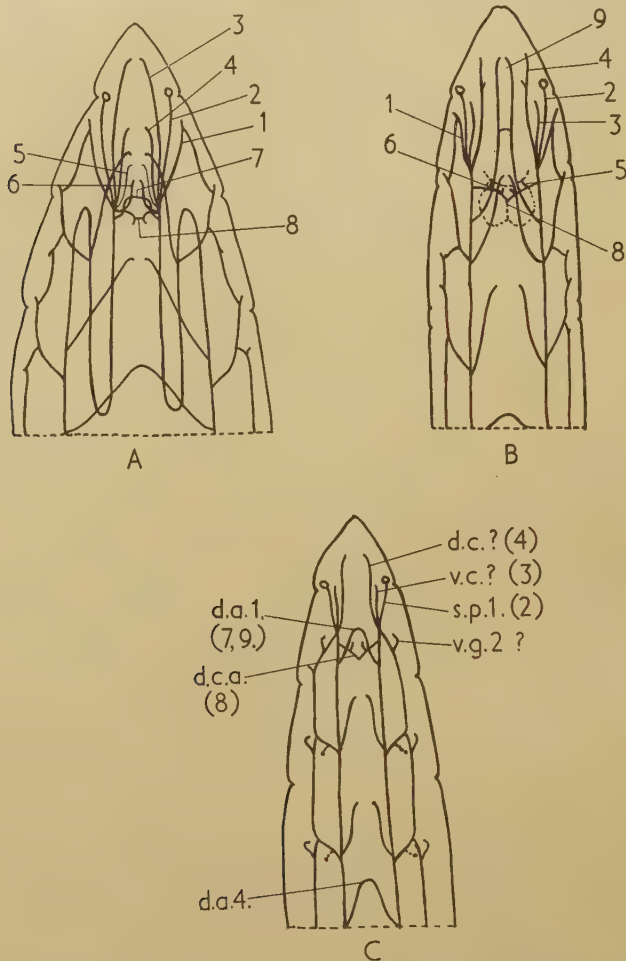


FIG. 9. A, the larval system of *Tabanus* sp. (after Stammer). B, the larval tracheal system of *Haematopota pluvialis* (after Cameron). C, diagrammatic reconstruction of the larval system of both *Haematopota* and *Tabanus*.

distribution of certain of the branches. . . . Stammer and I both agree that there are eight branches in the complex. . . . I have failed to trace his branch 7—a *cross commissure* joining the two tracheal trunks. . . . to an elongated branch distributed to the muscles at the anterior end of the head capsule, which was *not* mentioned by Stammer, I have assigned number 9. This branch is joined to its fellow by a *cross commissure* in segment II. Branch 9 which was not observed by Stammer probably represents the inner branch of segment II. It is joined to its fellow and in this

respect resembles the remaining members of the series of inner branches except those of segments III and IV already noticed. . . .'

It would appear that element 7 of Stammer—'a cross commissure'—is in fact the first *dorsal anastomosis* as also is Cameron's element 9, which he says was not observed by Stammer. The position of the elements may vary, although the origin and distribution remain constant. The nine pairs of inner branches correspond to dorsal anastomoses two to ten, so that with element 7 or 9 there are present, in all, tracheae corresponding to the *ten dorsal anastomoses*, and not nine as suggested by Cameron. The second and third do not join, but the component tracheae are present. Element 8 is undoubtedly the *dorsal cervical anastomosis*, the difference in the form of this in *Haematopota* and *Tabanus* possibly being a generic one: in both cases it gives rise to branches which pass to the supra-oesophageal ganglia. In order to interpret elements 3, 4, 5, and 6 it would be necessary to reinvestigate the material as too little information is given on the distribution of these elements. Almost certainly dorsal cervical, ventral cervical, and ventral ganglionic tracheal elements are present.

CONCLUSION

The impression gained from a study of the larvae discussed in this paper is that the Cyclorrhapha diverge *very little* from the generalized larval pattern. Further work is required on the systems of brachyceran species before any generalizations can be made: the scanty evidence at present available suggests that species from the few families studied also diverge little from the generalized larval pattern. This is not so in the remaining families of the Nematocera. In a future paper descriptions will be given of the tracheal systems present in representatives of these nematoceran families, and it will be shown that even these more anomalous systems can be interpreted in the light of the results given in the present paper.

I have to thank Professor V. B. Wigglesworth for his constant inspiration, Dr. John Smart for much helpful criticism, and Professor J. E. Smith for kindly reading through the manuscript.

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The Continuity between the Cavities of the Premandibular Somites and of Rathke's Pocket in *Torpedo*

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SUMMARY

The existence of an open communication between the cavity of Rathke's pocket and the cavity of the premandibular somite in *Torpedo ocellata* at the 7 mm. stage is demonstrated by means of microphotographs. They provide a complete refutation of the claim by B. Wedin that because he was unable to find continuity of open communication between the cavities of Rathke's pocket and of the premandibular somite, therefore E. S. Goodrich was mistaken in homologizing the preoral pit of *Amphioxus* with the hypophysis of craniates. On the contrary, Goodrich's observations are confirmed and his theory vindicated.

IN his paper on the connecting strands between the premandibular cavities and Rathke's pocket, B. Wedin (1951) stated that E. S. Goodrich (1917) and I (de Beer, 1925, 1926) had based his 'theory' that the preoral pit of *Amphioxus* was homologous with the cavity of the hypophysis or Rathke's pocket of craniates, on A. Dohrn's (1904) descriptions of embryos of *Torpedo*, and that he (and I) had misunderstood them. In particular, Wedin claimed that Dohrn's expression 'Verbindungen' in his description of the conditions in *Torpedo* had been mistranslated by Goodrich, and erroneously interpreted to mean that Dohrn had observed continuous hollow connexions between the cavities of the premandibular somites and that of Rathke's pocket. Wedin has re-examined Dohrn's material of *Torpedo* and found no continuous hollow connexions, but only strands of cells connecting the premandibular cavities with Rathke's pocket in which occasional lumina are present.

If Wedin had read Goodrich's and my papers with sufficient attention he would have seen that we based our 'theory' not on Dohrn's descriptions, but on our own positive observations in *Torpedo* of uninterrupted connexions between the cavities of the premandibular somites and of Rathke's pocket of which we published figures. We found this condition to be variable and the hollowness of the connexion transient. The discovery of the hollow connexion in *Torpedo* was made by Dr. Elizabeth Fraser in material belonging to J. P. Hill. This material was studied by Goodrich, and Dr. Fraser's observation confirmed and figured (Goodrich, 1917, text-fig. 3b, and pl. 28, fig. 15).

In my paper (de Beer, 1925, p. 103) I described observations that I had made on material of *Torpedo* which I had prepared myself, and wrote 'Zuerst stehen die prämandibular Somite und die Hypophyse in offener Verbindung', and gave a figure of a section of an embryo in text-fig. 17, illustrating this fact. In my book (de Beer, 1926) I repeated this in fig. 91, p. 72. Both Goodrich (1917, p. 548) and I (de Beer 1926, p. 35, fig. 31) were able to confirm the existence in *Anas* of a connexion with an obvious obliterated virtual lumen.

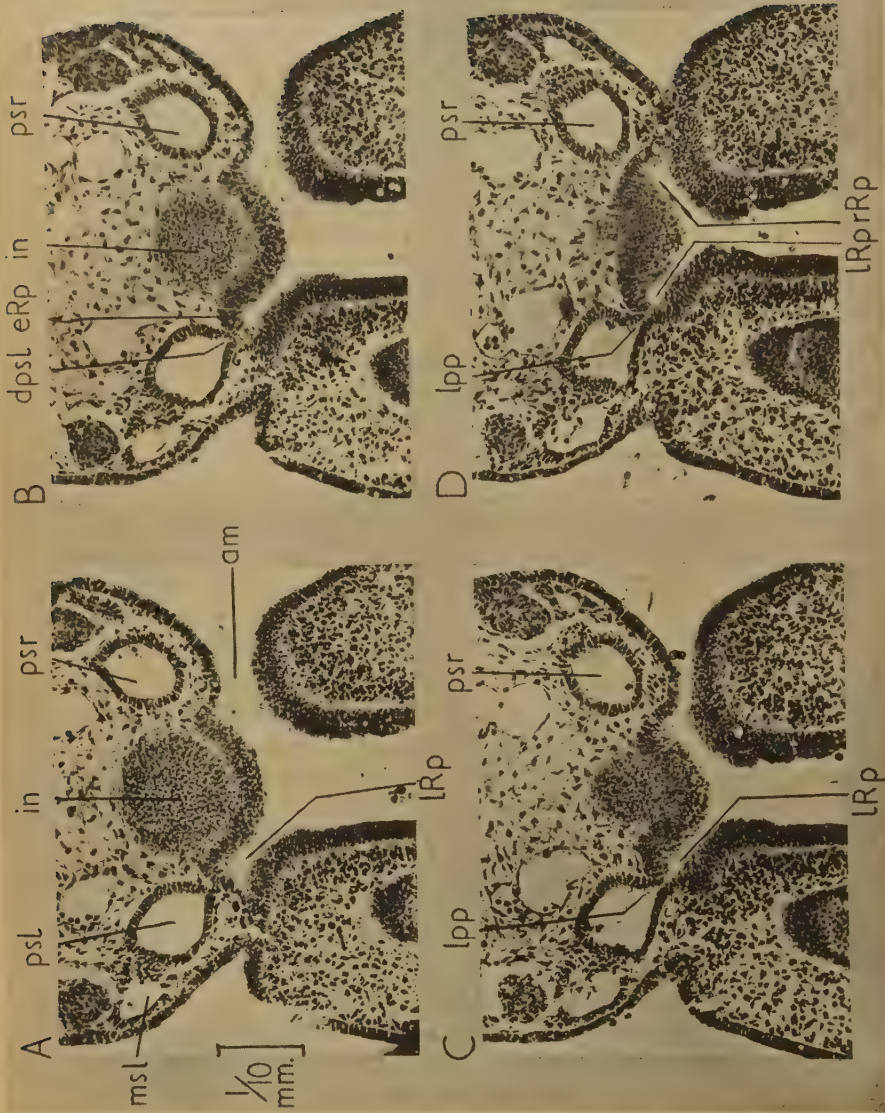


FIG. 1. A-H, microphotographs of eight consecutive sections through an embryo of *Torpedo ocellata*, 7 mm. long (series A, slide 1, row 4, sections 20-27. G. R. de B.—D.C.A., Oxford). Photos by M. G. Sawyers
 am, angle of mouth; cps, cellular strand connecting left and right premandibular somites; csl, cellular strand connecting left premandibular somite to epithelium of Rathke's pocket; csr, cellular strand connecting right premandibular somite to epithelium of Rathke's pocket; lpp, cellular strand connecting left premandibular somite to epithelium of Rathke's pocket; psr, cellular strand connecting right premandibular somite to epithelium of Rathke's pocket; LRp, cellular strand connecting left premandibular somite to epithelium of Rathke's pocket; dpst, cellular strand connecting left premandibular somite to epithelium of Rathke's pocket; erp, cellular strand connecting right premandibular somite to epithelium of Rathke's pocket.



somite to epithelium of Rathke's pouch; *dpsl*, depression in wall of right premandibular somite; *epsl*, epithelium of Rathke's pouch; *in*, posterior wall of infundibulum; *lpp*, open left proboscis pore; *lRp*, left diverticulum of Rathke's pouch; *msl*, cavity of left mandibular somite; *pc*, pharyngeal cavity; *psl*, cavity of left premandibular somite; *psr*, cavity of right premandibular somite; *rRp*, right diverticulum of Rathke's pouch; *Rp*, cavity of Rathke's pouch.

Dohrn's work was referred to by Goodrich together with that of other authors, because they had observed connexions between the premandibular somites and the hypophysis in *Torpedo*, *Raia*, *Phrynocephalus*, *Gongylus*, and *Anas*, and these connexions, whether solid or hollow, were obviously homologous with the hollow connexions which we have observed in *Torpedo*.

Wedin has been at some pains to prove that Dohrn's material shows no continuous hollow connexions between the premandibular cavities and the hypophysis, and has reproduced photomicrographs of four embryos of *Torpedo*, most of which show in the connecting strand of cells a lumen which Wedin asserts does not extend right through. I have no intention of contesting Wedin's descriptions of Dohrn's material. It is difficult to prove a negative proposition, and to invalidate Wedin's argument I only need to reproduce microphotographs of an embryo showing that the hollow connexion between the premandibular cavities and Rathke's pocket is a fact.

The embryo is one of *Torpedo ocellata*, 7 mm. long, cut into horizontal sections, 10 μ thick (series A, slide 1, row 4, sections 20–27 in the Department of Zoology and Comparative Anatomy, Oxford). Fig. 1, A, shows a section through the most anterior morphological level of the series. The posterior wall of the infundibulum is seen cut tangentially, in contact ventrally with the thickened epithelium of the anterior portion of Rathke's pocket. On each side of the infundibulum is a premandibular somite with a large cavity. The somite on the left is almost in contact with the epithelium of the left diverticulum of Rathke's pocket; that on the right is a little way distant.

Fig. 1, B, which is of the next section, 10 μ farther back, shows that the left premandibular somite has come into closer contact with the epithelium of Rathke's pocket and that the cavity of the somite shows a conical depression in its wall immediately over the point of contact.

Fig. 1, C, shows the open pore between the cavity of the left premandibular somite and the cavity of Rathke's pocket. The limiting membrane of the lining of the cavity of the somite is perfectly continuous with the membrane of the lining of the cavity of Rathke's pocket. The 'proboscis-pore' is open, and its diameter in the transverse plane is approximately 10 μ , which is about the same as its diameter in the sagittal plane, since the open pore is also plainly visible in the next section, shown in Fig. 1, D, at a level 10 μ farther back.

In fig. 1, E, the connexion between the left premandibular somite and Rathke's pocket is no longer open, but the wall of the right premandibular somite is seen to approximate to the epithelium of Rathke's pocket on that side. Rathke's pocket itself now appears as a closed cavity since the section is taken dorsally to its communication with the pharyngeal cavity.

Fig. 1, F, shows that the right premandibular somite is intimately connected with the epithelium of Rathke's pocket, and cone-shaped depressions can be seen in the walls of the cavity of the somite and of the right diverticulum of Rathke's pocket, immediately at the point of contact. The pore is not open on this side.

In the next section, fig. 1, G, the connexion between the right somite and

Rathke's pocket is still intimate, and the cone-shaped depression is also present in the wall of the cavity, but it is no longer seen in the wall of Rathke's pocket. In fig. 1, H, the right somite and Rathke's pocket are still in contact, and the strand can be seen connecting the left and right premandibular somites across the middle line.

In the light of these facts I challenge Wedin's statement (1951, p. 75): 'Leider machte Goodrich dabei einen Fehler und unterstellte Dohrn Beobachtungen, welcher dieser nicht gemacht hatte. Dieser Irrtum dürfte ausschlaggebende Bedeutung für die Gedanken Goodrich's haben.' Goodrich made no such mistake, nor that of drawing conclusions from facts which he had not observed himself. His hypothesis that the preoral pit of *Amphioxus* is homologous with the hypophysis of craniates, and that the open connexions between the premandibular cavities and Rathke's pockets represent 'proboscis-pores', remains unshaken. It might be added that it would still be unshaken if the connexions were solid, for it is a commonplace of comparative anatomy and of embryology that homologous structures may arise or remain solid, or hollow, and so no morphological significance can be ascribed to such differences.

In a subsequent paper, Wedin (1952) has described an open connexion between the cavities of the premandibular somite and of Rathke's pocket in *Torpedo*.

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The Optical Measurement of Depth

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SUMMARY

The formula for vertical measurement by means of the fine adjustment of the microscope,

$$n_2/n_1 \times \text{measured depth} = \text{real depth},$$

is true, where n_2 is the refractive index of the *object* and n_1 is that of the immersion medium of the *objective lens*. The refractive index of the mountant is unimportant.

Several additional complications of this method of measurement suggest that it should not be used unless it cannot be avoided, or rough figures are required quickly.

INTRODUCTION

THE measurement of depth in microscopical preparations has many applications, the most recent being the determination of refractive index, by means of the interference microscope, of cytoplasm and cell inclusions (for instance, Ross, 1954). This instrument permits measurement of the retardation of the light waves, and from this may be calculated the refractive index, provided that the thickness is known.

Optical depth measurements are taken by focusing successively the upper and lower surfaces of the object, and reading off the travel of the tube of the microscope by means of the calibration on the fine adjustment knob. Brattgard (1953) treats in detail of the accuracy with which these readings may be made.

The present paper is concerned with difficulties in calculating the actual depth from the readings so obtained, by consideration of the optical path.

CORRECTION OF DEPTH MEASUREMENTS

The accepted formula for obtaining depth is to multiply the distance through which the tube of the microscope must be moved to focus the upper and lower surfaces of the object, by the refractive index of the medium. It can be seen from the diagram that

$$\frac{\text{actual depth}}{\text{apparent depth}} = \frac{AC}{AB} = \frac{\tan \theta_2}{\tan \theta_1}$$

where AC is the real depth, and AB is the apparent depth, since $\angle ACD = \theta_1$ and $\angle ABD = \theta_2$. For small angles, $BA = BD$ and $CA = CD$, so that

$$\frac{\tan \theta_2}{\tan \theta_1} = \frac{\sin \theta_2}{\sin \theta_1}, \text{ and}$$

$$\frac{\text{actual depth}}{\text{apparent depth}} = \text{refractive index of the medium below the coverslip.}$$

[Quarterly Journal of Microscopical Science, Vol. 96, part 3, pp. 285-288, 1955.]

This is the usual formula found in elementary physics textbooks for the apparent depth of water seen with the naked eye, i.e. with small angles. The application of this formula to microscopy is seen in the paper by Addey (1922),

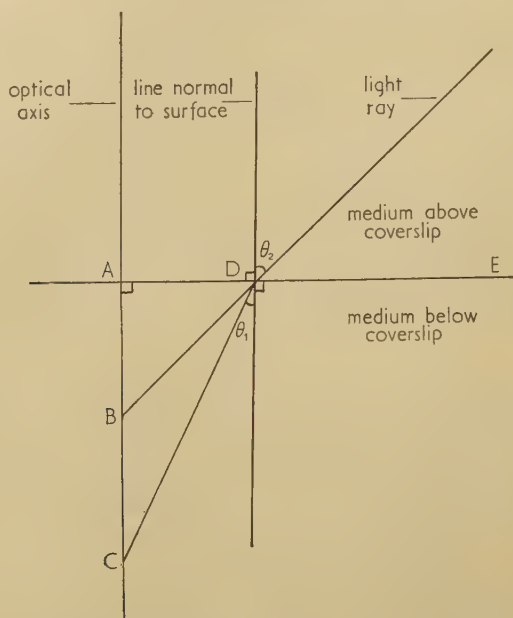


FIG. 1. Diagram showing the relationship between the apparent depth AB and the actual depth AC . For the sake of simplicity the upper side of the object is supposed to touch the coverslip. The coverslip is represented by a line AE because a flat plate at right angles to the optical axis does not affect the calculations.

in which appears the rather remarkable statement that 'If we consider a fairly narrow cone of rays, *such as we should be concerned with in microscopy*,

$$\frac{\tan \theta_1}{\tan \theta_2} = \frac{\sin \theta_1}{\sin \theta_2}$$

because the angles are small.' (The italics are mine.) θ_1 and θ_2 are again as in the figure, which is taken from Addey, and the formula is inverted because Addey approached the problem differently.

At first, then, it appears that we should use a correction, based not on the sines of the angles, as the refractive index correction is, but on their tangents.

LENS CORRECTIONS

Calculation shows that the correction will not be the same figure for all angles, but will increase from the refractive index figure at the paraxial rays, as shown in table 1, where the refractive index of the medium below the coverslip is taken as 1.52.

This is of course equivalent to spherical aberration of the objective lens, due to the medium in which the object is mounted.

Now, lens corrections for spherical aberration take the form of approximating the marginal rays to the paraxial focus, so that the paraxial rays alone need to be considered; and for these, which are of small angle, the refractive index correction applies. It is evident, therefore, that the right correction is used, but for the wrong reason.

TABLE I

θ_2	θ_1	Correction factor: $\frac{\tan \theta_2}{\tan \theta_1}$
5°	3° 17'	1.524
10°	6° 34'	1.531
20°	13° 0'	1.577
30°	19° 12'	1.659
40°	25° 2'	1.797
50°	30° 16'	2.042
60°	34° 44'	2.499
70°	38° 12'	3.491
80°	40° 23'	6.688

REFRACTIVE INDICES

When applying the correction, the refractive index in question is that of the substance through which the rays travel a different distance on altering the focus; that is, the reading should be multiplied by the refractive index of the *object*, not of the mounting medium. For biological tissues in balsam, the difference is roughly 0.1%, and since Brattgard gives the accuracy of measurement as $\pm 0.1 \mu$, this would be unimportant except for objects of about 100 μ or more in thickness.

For cells in saline, however, the difference is roughly 1.0% (see Ross's figure of 1.3535 for the cytoplasm of the spermatid of *Locusta*). The difference is therefore greater than the error of the readings for objects of about 10 μ and upwards.

It is known that this correction should not be applied for homogeneous immersion systems, or alternatively the more general formula may be applied, by multiplying by n_2/n_1 , where n_2 is the refractive index of the object, and n_1 is the refractive index, not of the mountant (for example, Canada balsam), but of the immersion medium (lens immersion oil, water, or air). This is not quite clear in Brattgard's paper.

It is important to realize that the movement of the tube of the microscope in focusing the top and bottom of the object is equal to the thickness of the object only if the refractive index of the lens immersion medium is identical with that of the object.

SPHERICAL ABERRATION

Another source of error arises from the fact that the spherical correction is calculated for a particular thickness of intervening substance, i.e. coverglass and medium, and vertical measurements therefore necessarily involve using

the lens at depths for which it is wrongly corrected. The tube length can be adjusted to give spherical correction for only one of the two foci. If the tube length be adjusted for each in turn, then an additional correction will be required, involving the tube lengths and the focal length of the objective lens.

Corrections are calculated to bring the marginal rays to the paraxial focus, with which the position of the best focus does not quite coincide. When the microscope is racked up and down, the best focus moves in relation to the paraxial focus, in a way that depends on the characteristics of the particular objective lens in use. The error, however, is likely to be small for small vertical travel, only assuming measureable proportions in the approximate region of 100 μ .

DISCUSSION

A dilemma therefore arises, because to measure the thickness it is necessary to know the refractive index, and to calculate the refractive index from the wave retardations obtained by the interference microscope, it is necessary to know the thickness.

Therefore it is suggested that whenever possible, it is preferable to make horizontal measurements, either of the same object if it is spherical or cylindrical, or of the similar objects lying in a different orientation—for instance on a differently cut section.

In this paper the object has been regarded for simplicity as a plane, parallel-sided layer. Refraction at the surfaces of curved objects complicates measurement still more, and adds weight to the above suggestions, because it is obvious that all rays leaving the bottom of a spherical or horizontal cylindrical object must pass through it if they are to reach the objective. The rays will therefore be refracted at the upper surface of the object, and in the latter case will be refracted differently in the two different axes.

In the special case of the measurement of an object which approximates to a vertical line, for example a flagellum, n_2 should be taken as the refractive index of the *mountant*, i.e. saline or Canada balsam, since rays from the bottom of it will not pass through the object itself.

My thanks are due to Dr. J. R. Baker for his advice and suggestions, and to Mr. T. A. Minns of Messrs. Watsons Ltd. for a valuable discussion. The paper arose in connexion with research carried out under a grant from the Medical Research Council.

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A Study of the Argentaffin (Kultschitzky) Cells in frozen-dried Tissue by Phase-Contrast Microscopy and Ultra-Violet Light

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With one plate (fig. 1)

SUMMARY

1. The argentaffin cells in guinea-pig intestine have been studied by phase-contrast microscopy and in ultra-violet light. Frozen-dried tissue has been used.

2. One such cell in a section $5-7\mu$ thick was selected and studied throughout. In an unfixed section mounted in nonane, the cytoplasm is packed with fine granules which emit a greenish-yellow fluorescence in ultra-violet light of wavelength $2,750\text{ \AA}$. There is also photographic evidence of absorption of light of this wavelength.

3. After formaldehyde fixation the fluorescence changes to orange-yellow and becomes much weaker. The absorption of light by the granular cytoplasmic contents is also greatly reduced.

4. There is no fluorescence and no photographic evidence of absorption of light of wavelength 2570 \AA by the granular cytoplasmic contents, either before or after formaldehyde fixation.

5. The nuclear chromatin pattern in these cells is unaltered by formaldehyde fixation, and is well demonstrated in photographs taken in ultra-violet light of both the wavelengths mentioned above.

INTRODUCTION

IT has recently been reported that the granular material in the argentaffin (Kultschitzky) cells is a formalin artifact (anon., 1954). Also, Eros (1932) reported that the granules in formaldehyde-fixed tissue fluoresce in ultra-violet light. This phenomenon was re-examined by Jacobson (1939) in the case of formaldehyde-fixed human argentaffin cell (so-called 'carcinoid') tumour, the maximum amount of absorption being in the region of $2,700\text{ \AA}$. Lison (1953) stated that the yellow fluorescence is only obtained after formaldehyde fixation.

Erspamer and Asero (1952) claimed that the substance 'enteramine' within the argentaffin cells is 5-hydroxytryptamine. They, employing the picrate derivative, and Rapport, Green, and Page (1948), and Rapport (1949), working with the tryptamine-creatinine-sulphate complex, studied the ultra-violet absorption spectrum and found that each compound shows maximal absorption at $2,750\text{ \AA}$. They did not employ formaldehyde at any stage. It is therefore important to ascertain whether, in fact, the granular argentaffin substance does or does not fluoresce in ultra-violet light and absorb such a wavelength, without previous treatment with formalin.

[Quarterly Journal of Microscopical Science, Vol. 96, part 3, pp. 289-293, 1955.]

Although the granular material within these cells in human and guinea-pig material is fixed by osmium tetroxide (Christie, 1955) and by potassium dichromate (unpublished observation), it was considered advantageous to avoid chemical fixatives if possible. Frozen-dried tissue was the obvious choice, provided the granular material is retained in the unfixed cell in sections thus prepared. This was found to be the case, and a study of the argentaffin cells both by phase-contrast microscopy and in ultra-violet light will be the subject of the present communication. First, cells were studied in unfixed tissue; then the same cells were re-examined after formaldehyde fixation, and finally stained by Gomori's (1948) hexamine silver nitrate technique to ensure correct identification of the cells under examination.

MATERIAL AND METHODS

After a guinea-pig had been killed instantly, a piece of duodenum (or upper jejunum) was transferred to liquid propane at -185°C . within 45 seconds. Frozen-dried material was obtained by drying the tissue at -40°C . for 3 days and then embedding in paraffin wax at 58°C . over a period of 2 minutes. Sections $5-7\mu$ thick were cut and mounted on quartz slides. After removal of the paraffin by flooding the section with nonane, a quartz coverslip was applied and sealed round the edges with molten paraffin wax in order to prevent the nonane from evaporating. The following procedures were then carried out:

(1) Examination under ordinary light, first by ordinary and then by phase-contrast microscopy, and finally by ultra-violet light of wavelengths $2,750\text{ \AA}$ and $2,570\text{ \AA}$.

(2) After removing the coverslip carefully and allowing the nonane to evaporate, the section was exposed to formaldehyde vapour (from a solution of commercial formalin of 40% strength in a Coplin jar) for 4 hours and then immersed in 10% formaldehyde with 1% calcium chloride for 16 hours. After washing in distilled water for half an hour the section was remounted in nonane and examined as in (1) above.

(3) The coverslip was again removed and the section stained by Gomori's (1948) hexamine silver nitrate technique for approximately 18 hours, when the granules in the argentaffin cells appeared a light brown colour against a practically unstained background.

RESULTS

In frozen-dried unfixed paraffin sections of guinea-pig small intestine, the argentaffin cells are clearly visible under ordinary light microscopy, but are even more clearly discernible when phase-contrast is used. In fig. 1, A a plump, roughly spherical cell can be seen filled with fine granules. On formaldehyde fixation and subsequent staining it is seen to contain the silver-reducing granules typical of argentaffin (Kultschitzky) cells, a confirmation of its identity.

Under ultra-violet light of wavelength $2,750 \text{ \AA}$ this cell emits a clearly visible greenish-yellow fluorescence, and contains cytoplasm which strongly absorbs light of this wavelength (fig. 1, B).

After formaldehyde fixation this cell shows some shrinkage (fig. 1, D), but no more than is apparent in nearby cells. However, when viewed under ultra-violet light of wavelength $2,750 \text{ \AA}$ there is a considerable difference in the cell's appearance. The bright greenish-yellow fluorescence has now changed to orange-yellow and its intensity is considerably diminished. Fig. 1, E shows that the intensity of absorption of light of this wavelength by the granular cytoplasmic contents is now not detectable photographically.

The reduction of the intensity of both the fluorescence and the absorption after formaldehyde fixation could possibly be explained on the basis of alterations in the intensity of the ultra-violet light source overnight (for a lapse of this time is required for formaldehyde fixation to be accomplished). To overcome this possible source of error, sections from the same paraffin block of frozen-dried unfixed material were cut and examined in conjunction with the formaldehyde fixed ones at the same time. Over a period of about 15 minutes the sections, fixed and unfixed, were repeatedly interchanged and examined by myself and two colleagues, without knowledge as to which one was fixed and which not so treated, in order to eliminate the possibility of personal factors prejudicing decisions as to colour and intensity changes, as well as eliminating errors due to changes in the intensity of the light source. A change in both the colour and the intensity of the fluorescence after formaldehyde fixation was clearly apparent.

The unfixed cell was also examined in light of wavelength $2,570 \text{ \AA}$, both before and after formaldehyde fixation. Fig. 1, F depicts the same cell after, and fig. 1, C the cell before formaldehyde fixation, when photographed in light of this wavelength. In both figures strong absorption by the nuclear chromatin is shown, but not by the granular cytoplasmic contents; nor do they fluoresce.

There is also absorption of light of wavelength $2,750 \text{ \AA}$ by the nuclear chromatin, and comparison of fig. 1, B and 1, C with 1, E and 1, F show that formaldehyde fixation has not materially altered the typical nuclear pattern; or rather, since the pattern was first depicted in formaldehyde-fixed tissue as, for example, in Ciaccio's (1906) illustration, it is more correct to say that the characteristic pattern is not produced by such fixation.

The granules have the same appearance and are of about the same size in unfixed and fixed tissue, when studied by phase-contrast microscopy, as can be seen from fig. 1, A and 1, D.

DISCUSSION

Gomori (1948) maintained that formalin either alone or in mixtures is essential for the fixation of the argentaffin cell with its content of cytoplasmic granular material. On the other hand, Cordier (1926) reported that he could see and stain the granules with neutral red, in teased fresh tissue suspended in 'sérum artificiel'.

It has now been shown that, in frozen-dried guinea-pig tissue, the granules are present in a morphologically similar form to those seen in formaldehyde-fixed tissue. In such unfixed tissue sections prepared by the Altmann-Gersh technique they also emit a greenish-yellow fluorescence when viewed in ultra-violet light of wavelength $2,750 \text{ \AA}$. After formaldehyde fixation the colour changes to orange-yellow and the intensity is diminished. The intensity of light absorption as assessed by photographic means is also greatly diminished by treatment with formalin. In the cell depicted in fig. 1, E it was not sufficient to affect the photographic plate, though in other argentaffin cells examined in the same and in other sections there was often slight darkening of the cytoplasm. Thicker sections, such as can be employed when one is examining carcinoid tumours, would almost certainly show considerable absorption amongst groups of cells rich in granular material; but throughout this work only single cells in sections $5\text{--}7\mu$ thick have been examined.

These observations are opposed to the hypothesis that the granular material is a formalin artifact, if by this is meant that in tissue fixed in fixatives not containing formalin the granules are not demonstrable. However, they confirm that formalin almost certainly has some chemical action on the granules—a deduction previously made from the observation that previous fixation by formaldehyde prevents the subsequent darkening of them by osmium tetroxide (Christie, 1955).

It appears that the above findings favour the hypothesis of Cordier (1926), who also worked with guinea-pig material, that the argentaffin substance is present in granular form in fresh tissues examined immediately after removal from the animal.

I should like to thank Mr. R. King for taking the photographs, and Miss Shirley Charter for preparing the frozen-dried sections.

FIG. 1 (plate). A, a frozen-dried, unfixed, and unstained paraffin section of guinea-pig duodenum, showing a crypt of Lieberkühn with a relatively large, spheroidal argentaffin cell containing cytoplasm packed with fine granules. (Phase-contrast.)

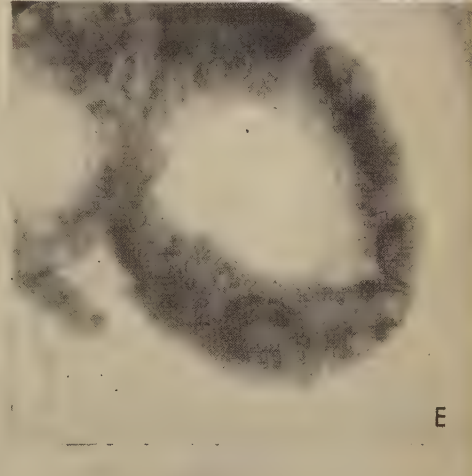
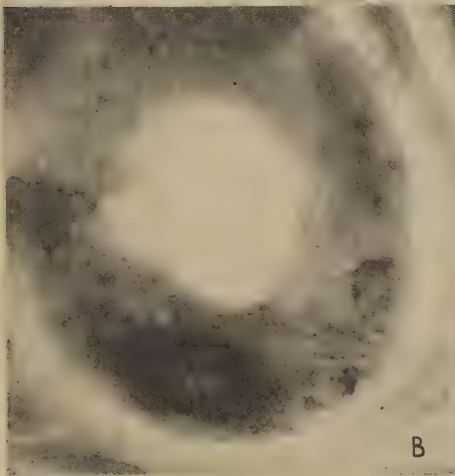
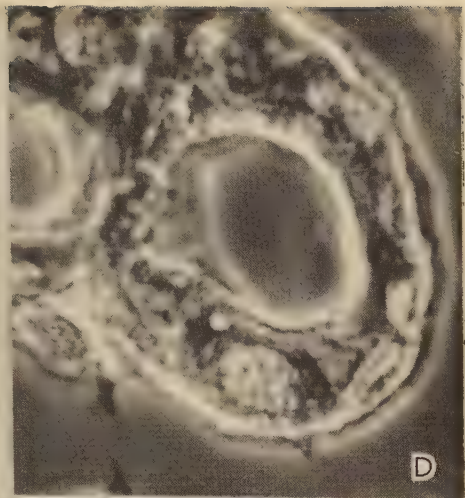
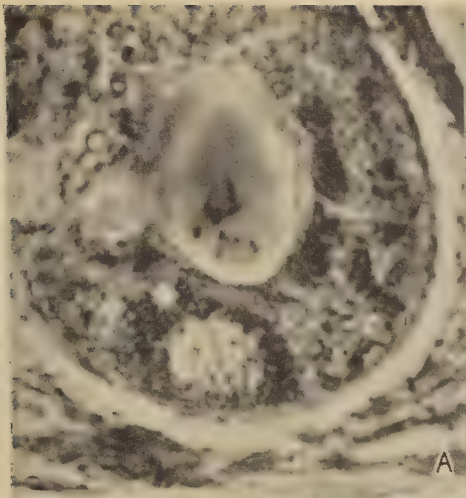
B, the same crypt of Lieberkühn as shown in fig. 1, A, viewed by ultra-violet light of wavelength $2,750 \text{ \AA}$. The cytoplasmic contents of the argentaffin cell show strong absorption of this light. Although it is better seen in the next figure, the typical chromatin pattern within the nucleus can be discerned.

C, the same crypt as in fig. 1, A, B, photographed in ultra-violet light of wavelength $2,570 \text{ \AA}$. Although there is still strong absorption by the nuclear chromatin, the cytoplasmic contents of the argentaffin cell do not absorb light of this wavelength.

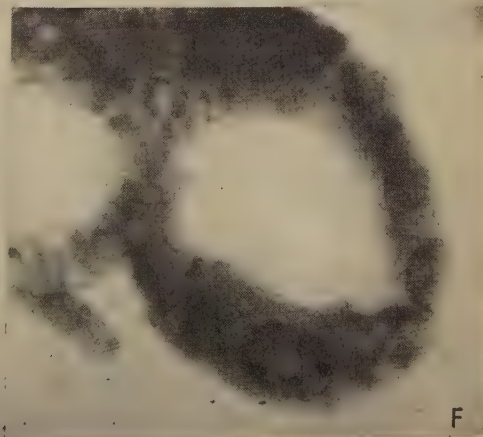
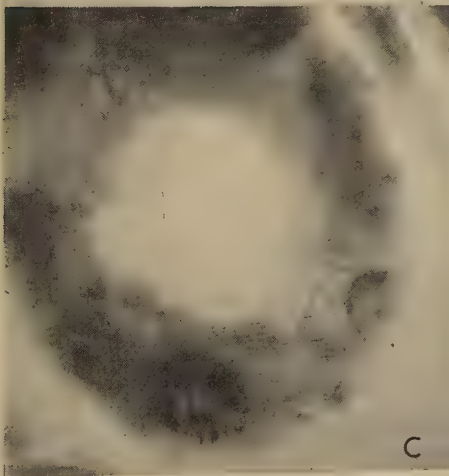
D, the same crypt viewed by phase-contrast microscopy after formaldehyde fixation. Apart from considerable shrinkage, the cytoplasmic contents of the argentaffin cell have been otherwise unaltered by fixation.

E, the same crypt viewed by ultra-violet light of wavelength $2,750 \text{ \AA}$, after formaldehyde fixation. Although the nuclear chromatin still shows strong absorption and reveals a pattern unchanged by fixation, the cytoplasmic contents now show no photographically detectable absorption.

F, the same crypt, now photographed in ultra-violet light of wavelength $2,570 \text{ \AA}$, after formaldehyde fixation. Nuclear chromatin still shows strong absorption, but, as in fig. 1, C, there is no cytoplasmic absorption by the argentaffin cell.



10 μ



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A Study of the Kultschitzky (Argentaffin) Cell with the Electron-microscope, after Fixation by Osmium Tetroxide

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With 2 plates (figs. 1 and 2)

SUMMARY

1. The Kultschitzky cells of the alimentary canal of man and the guinea-pig were studied with the light-microscope, buffered osmium tetroxide being used as fixative.

2. The Kultschitzky cells of the guinea-pig were shown by the electron-microscope to contain spheroidal granules having a maximum diameter of 0.3μ . These granules are considerably darkened by fixation for only 4 hours in 1% osmium tetroxide solution.

3. The opinion that the granules of the Kultschitzky cell are only an artifact of formaldehyde fixation is denied.

INTRODUCTION

IT has recently been claimed that the granular material present in the Kultschitzky (argentaffin) cells of the mammalian alimentary canal is an artifact of formaldehyde fixation (anon., 1954). That formaldehyde is essential for the fixation of these cells is also implied in the observation of Gomori (1948) that the granules are preserved by all aqueous fixatives containing formaldehyde but are dissolved by all formaldehyde-free aqueous fluids.

Nussbaum (1879) and Grutzner and Menzel (1879) demonstrated in the stomach and pylorus of the dog, cells which contain granular material that is darkened by fixation in osmium tetroxide. From their figures it is apparent that they were describing cells similar to those described by Kultschitzky (1897), and now often called argentaffin cells because they reduce ammoniacal silver nitrate (Gosset and Masson, 1914).

Buffered osmium tetroxide, introduced by Palade (1952), is now considered the best fixative for work with the electron-microscope; other fixatives, known to give good results with the light-microscope, prove deficient under the immense resolving power of this instrument (Porter, Claude, and Fullam, 1945).

It was considered worthwhile to repeat the earlier work, but to buffer the osmium tetroxide solution used for fixation and to employ the electron-microscope as well as the light-microscope.

MATERIAL AND METHODS

Pieces of the sigmoid colon and appendix of man, removed at operation, and of the duodenum of the guinea-pig were fixed in freshly-made 1%

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osmium tetroxide solution, buffered at pH 7.3–7.5 with 0.028 M. sodium acetate / sodium veronal buffer, for periods ranging from 4 to 36 hours.

After washing thoroughly in running tap water, small pieces of tissue were embedded in gelatine, and frozen sections approximately 8–10 μ thick were cut without hardening the gelatine block in formalin. This procedure is difficult owing to the softness of the gelatine, and requires the cutting of many sections to obtain one suitable for cytological examination. The sections were mounted in Farrants' medium.

For electron-microscopy pieces of guinea-pig duodenum, fixed for 4 hours and thoroughly washed in running tap water, were embedded in ester wax (Steedman, 1947). Sections approximately 0.2 μ thick were cut and examined. Sections similarly prepared but 5–7 μ thick were made and examined unstained with the light-microscope in order to give familiarity with the appearance of these cells in sections prepared in this way.

RESULTS

The cells about to be described are morphologically identical with the Kultschitzky (argentaffin) cells described by Masson (1928) and Cordier (1926) in the intestine of man and the guinea-pig, and they occupy the same position in the crypts of Lieberkühn. Their identity is thus established. The histochemical properties of these cells have been discussed by Lison (1953) and Pearse (1953). Cordier (1926) considered a combination of the argentaffin and chromaffin reactions as sufficient for their identification.

Fig. 1, A and B, shows morphologically typical argentaffin cells in unstained gelatine sections of human and guinea-pig material respectively, after fixation in osmium tetroxide. Other sections of tissue, appropriately fixed, show morphologically similar cells situated at the bases of the crypts of Lieberkühn in the small intestine and at the bases of the crypts of the large intestine: such cells give the histochemical reactions for the argentaffin cells.

In both species the cells contain masses of small granules. These are infra-nuclear in man: that is, they are confined to the region between the nucleus and the basement membrane. Besides being fixed, the granules are also darkened by osmium tetroxide, or even blackened if fixation is sufficiently prolonged.

Sections of tissue fixed for 4 hours in buffered 1% osmium tetroxide, embedded in either paraffin or ester wax, and cut 5–7 μ thick, show a moderate amount of darkening of the granules. On the other hand, sections of formaldehyde-fixed tissue subsequently treated with osmium tetroxide for prolonged periods (several days) show no darkening of the granules, whereas lipid tissue

FIG. 1 (plate). A, human sigmoid colon, obtained at operation, fixed in buffered 1% osmium tetroxide for 16 hours. An argentaffin cell, with characteristically situated, blackened, infra-nuclear granules is present. Unstained gelatine section.

B, guinea-pig duodenum fixed in buffered 1% osmium tetroxide for 36 hours. Two argentaffin cells packed with numerous small dark granules are present. Unstained gelatine section.

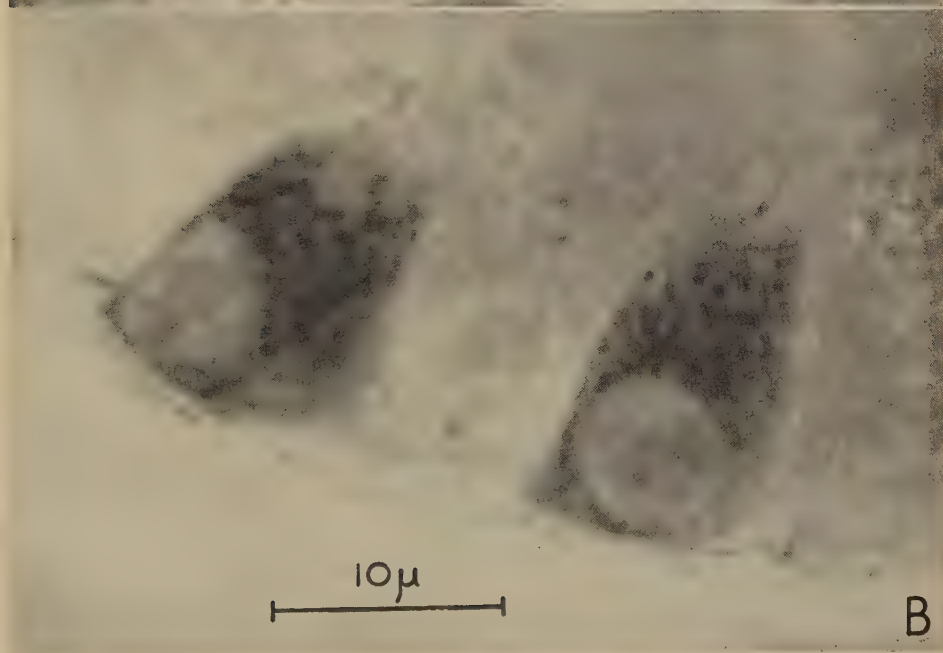


FIG. 1
A. C. CHRISTIE



FIG. 2
A. C. CHRISTIE

(e.g. adipose tissue in the adjacent mesentery) is blackened after an hour's exposure or even less.

Fig. 2 is an electron-micrograph of an argentaﬀin cell at the base of a crypt of Lieberkühn in guinea-pig duodenum. In the section, only about 0.2μ thick, the nucleus is not shown. The cytoplasm contains numerous, more or less evenly dispersed, oval but almost circular bodies, the largest being approximately 0.3μ in their longest diameter. As they are all seen to be of the same shape in the plane of the photograph, it is most likely that they are nearly spherical; for otherwise, provided that they are not all arranged parallel to one another at right angles to the plane of the section (and of the photograph)—and this is very unlikely—not all of them would appear of the same shape, as in fact they are. A contrast is provided by the mitochondria in the nearby cells in fig. 2: these, known to be elongated in intestinal epithelial cells fixed in fixatives containing osmium tetroxide, are seen assuming various elongated shapes, only occasionally being circular when, by chance, a true cross-section has been made by the microtome-knife.

All the spheroidal bodies are not of the same size. This disparity may be fallacious, as the section is of about the same thickness, or even thinner than that of many of the granules; consequently some will not have been sliced through their centres, and thus will appear smaller, though still oval. In the absence of serial sections it is impossible to settle this point merely by examination of a single two-dimensional photograph.

Some of the spheroids have particulate condensations of reduced osmium approximately evenly dispersed just beneath their margins. This phenomenon is likely to be an artifact. Otherwise the granules have no structural characteristics.

In other electron-micrographs the nuclear pattern was studied and found to be identical with that in formaldehyde-fixed tissue, consisting of an open, so-called 'vesicular' arrangement of chromatin threads, with aggregations of chromatin more or less evenly spaced round the periphery. This pattern was depicted by Ciaccio (1906) in formaldehyde-fixed tissue.

DISCUSSION

It is thus apparent that osmium tetroxide not only fixes but clearly depicts the granules in the argentaﬀin cells without the necessity of employing subsequent staining techniques. The granules are as clearly defined and of about the same size as in formaldehyde-fixed tissue stained by the conventional methods (enumerated by Lison, 1953).

For electron-microscopy it is necessary to use osmium tetroxide, suitably buffered, in order to obtain a picture for critical study. The granules, thus

FIG. 2 (plate). An electron-micrograph of the base of a crypt of Lieberkühn in the duodenum of a guinea-pig, showing an argentaﬀin cell containing numerous dark spheroidal granules. In the cytoplasm of adjacent cells mitochondria are visible, some showing transverse striations.

fixed, are present as spheroidal (almost spherical) bodies, probably all of about the same size (0.3μ), although conclusive evidence on this point is not obtainable from examination of single sections. No characteristic structural pattern is apparent apart from fine peripheral deposits of reduced osmium in some of them; this is possibly an artifact.

These findings demonstrate that formaldehyde, alone or in mixtures, is not essential for demonstrating the granular material within these cells. If the granularity of the material is an artifact, then it is misleading to refer to it as an artifact of formaldehyde fixation, for that would imply that this agent is the only substance capable of producing granules within these cells.

Further, of all fixatives investigated by Strangeways and Canti (1927), osmium tetroxide was found to produce the least change as compared with the living cell. It is generally regarded as an excellent preservative of the living structure of cells. If the granules are an artifact produced by formaldehyde, it is surprising that apparently identical granules should be produced by such a reliable fixative as osmium tetroxide.

Formaldehyde is known to possess the property of polymerizing phenolic compounds (Megson, 1936), and this could be the case with the argentaffin granules if these were only present after formaldehyde fixation, for the argentaffin substance has been considered to possess a phenolic radicle (Cordier and Lison, 1930). However, such an action on the part of osmium tetroxide has never been reported.

Recently the argentaffin granules have been shown to contain phospholipid (Christie, 1954). It is known that osmium tetroxide fixes lipids, at the same time being reduced by many of them, though not as energetically as by most triglycerides (Baker, 1950). Whilst fats are blackened by post-fixation in osmium tetroxide, the argentaffin substance is not. It is interesting to note that Grutzner and Menzel (1879) made the deduction that the blackening of the granules when fixed in osmium tetroxide is due to the former's strong reducing properties: it was, in fact, the first histochemical observation recorded concerning these cells.

I am indebted to the surgical staff of the Royal Cancer Hospital for their ready help in providing me with suitable surgical material. I am also grateful to Mr. M. S. C. Birbeck for undertaking the work with the electron-microscope, and to Mr. F. E. Speed, also of the Chester Beatty Research Institute, for the microphotographs.

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Experiments on the Mechanism of Silver Staining

III. Quantitative Studies

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SUMMARY

The quantitative aspects of silver staining of sections have been investigated with radioactive silver (Ag^{111}). The concentrations of reducible silver, developed silver, and silver nuclei in the sections were determined, but it is doubtful if the values obtained for silver nuclei are significant.

All three forms of silver increased with pH, time, and the concentration of silver in the impregnating solution. Temperature of impregnation had little effect on the uptake of reducible silver, but increased the developed silver, presumably by increasing the silver nuclei. An increase in the temperature of a hydroquinone-sulphite developer increased the amount of reducible silver reduced by the developer. The deposition of silver by a glycine physical developer was shown to follow a curve which was reasonably consistent with the assumption of a typical autocatalytic reaction.

The uptake of silver by non-nervous tissues provided evidence that the process is not specific for nerves; the final specificity of staining is determined during development.

The quantitative results are consistent with the hypothesis that the histidine in the sections is responsible for the combination of reducible silver.

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INTRODUCTION

IN previous papers (Peters, 1955, *a* and *b*), various aspects of the impregnation and development processes in the silver staining of nervous tissue were considered. It was shown that during the impregnation of sections of fixed tissue in solutions of silver nitrate, two essentially different reactions take place between the silver ions and the sections. Most of the silver is combined with the sections in an unreduced, but reducible, state; it was suggested that this silver fraction was combined with the histidine in the sections. A smaller

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fraction of silver is reduced to form silver nuclei. While the combination of unreduced silver is a rapid process, and is complete within 15 minutes at 37° C. and pH 9, the formation of silver nuclei is a slower process, so that at least 2 hours' impregnation is necessary to form sufficient silver nuclei to give rise to a good staining picture on development. During development, the silver nuclei act as centres for the deposition of additional silver derived from the action of the developer on the reducible silver fraction.

The results of the earlier staining experiments were determined visually, on the basis of the depth of staining produced after a series of variations in the impregnating stage. The present paper deals with a quantitative study of impregnation and development. A preliminary investigation was made in which the silver deposited on to the sections after development was determined by a micro-Volhard titration (Wigglesworth, 1937); but this method was not accurate enough for the present purposes and could not be used to determine very small concentrations of silver. Therefore, in the experiments to be described here, radioactive silver (Ag^{111}) was employed.

METHOD

Radioactive silver, Ag^{111} , was obtained from A.E.R.E., Harwell, where it is produced by neutron bombardment of palladium sponge ($\text{Pd}^{110} (n, \gamma) \rightarrow \text{Pd}^{111} \beta^- \rightarrow \text{Ag}^{111}$). Samples of 0.5 g. of palladium sponge were used, and the silver was extracted with warm 25% nitric acid. After extraction, the undissolved palladium was filtered off and washed with distilled water to extract as much silver as possible. The filtrate was then diluted with water to 200 ml., and 1 g. of 'Analar' silver nitrate was added to the solution as a 'carrier'. During the extraction, some palladium, including Pd^{109} which is produced as a side-product in the formation of Ag^{111} , was dissolved by the nitric acid, so that the solution had a faint yellow colour, but as far as could be determined histologically, this palladium did not interfere with the silver staining.

The concentration of silver in this stock solution was determined at the end of the experiment, when the radioactive silver had decayed, by a Volhard titration. In the experiments to be described, the concentration of the silver nitrate in the stock solution was 0.038 M.

Sections of rat spinal cord and human cerebrum were used in these experiments. A few initial experiments were carried out with frog spinal cord, but the very low count obtained after staining these small sections placed a serious limit on the number of experiments which could be carried out before the silver had decayed to a small counting value.

Sections were cut at 10μ and impregnated while floating free in a 3.8×10^{-4} M (approx. 1/20,000) solution of silver nitrate; the solutions were brought up to the required temperature for impregnation before the sections were added. The solutions were buffered at pH 9.0, 8.0, and 7.0 with boric acid / borax buffer, and at pH 5.6 with acetic acid / sodium acetate buffer. The final concentration of buffer in the impregnating solutions was 0.01 M.

After impregnation, sections were removed from the solution with a glass rod, rapidly rinsed in distilled water, and treated in one of four ways:

- (1) With 2% hydroquinone—to determine the *total silver* taken up during impregnation, i.e. reducible silver plus silver nuclei (see Peters, 1955a).
- (2) With 1% hydroquinone in 10% sodium sulphite—to determine the *developed silver*, i.e. silver reduced by the developer plus silver nuclei.
- (3) With 2% sodium sulphite for 5 minutes to determine the *silver nuclei* (Samuel, 1953).
- (4) With citric acid / sodium citrate buffer at pH 3.2 for 1 hour to determine the *silver nuclei* (see Peters, 1955a).

Unless otherwise stated, all determinations of silver nuclei were made after removal of the reducible silver with the citrate buffer.

After treatment, the impregnated sections were thoroughly washed with distilled water, transferred to counting disks, and dried in an oven at 100° C. The mean volumes of the sections used in any experiment were calculated from the surface area and the thickness (10μ). The concentration of silver in a section was calculated from the volume of the section, the count produced by the Ag^{111} in the section, and the count produced by control disks with a known concentration and volume of the original silver nitrate solution. The approximate standard deviation of any count never exceeded 5%.

The half-life of the Ag^{111} was calculated after the Pd^{109} had decayed, and was found to have a value of 7.35 days, which compares satisfactorily with the Harwell figure of 7.5 days. Initial experiments showed that the Pd^{109} , which has a half-life of 13 hours, had become negligible in the control disks after 120 hours, while the Ag^{111} could still be counted up to 700 hours. However, on the disks carrying the impregnated sections, the Pd^{109} did not become negligible until 200 hours; these disks were not counted for silver until the Pd^{109} had completely decayed. It was calculated from the sections which had been treated with 2% hydroquinone that the Pd^{109} concentration in the sections was approximately 7 times at pH 9, 2.5 times at pH 7, and 60 times at pH 5.6, that of the Pd^{109} in the impregnating solution. It is apparent from these figures that the uptake of palladium bore no relation to that of silver, and since the concentration of palladium in the impregnating solution was very low compared with that of the silver, it is considered that it had little or no effect on the silver uptake.

RESULTS

All results have been expressed as g. silver/ml. of tissue.

(1) *The effect of pH on silver uptake*

Sections of alcohol-fixed rat spinal cord were impregnated in a 3.8×10^{-4} M solution of silver nitrate for 16 hours at pH 9.0, 8.0, 7.0, and 5.6. The solutions were buffered in the manner already described. After impregnation, sections were treated to determine the total and developed silver; silver nuclei were

determined both with the sodium sulphite and citrate buffer. The results are shown in fig. 1, in which the 'grand means' of a number of experiments have been plotted against the corresponding pH values. More detailed results are given in table 1.

It can be seen that the concentrations of all types of silver increase with an increase in the pH of the impregnating solution and that there is a rapid increase in the silver uptake from pH 8 to 9. This quantitative result confirms that observed in the earlier staining experiments (Peters, 1955*a*).

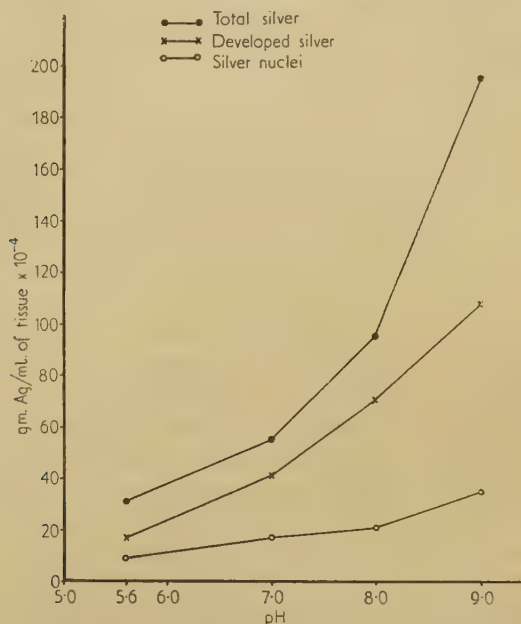


FIG. 1. The effect of pH on the uptake of silver at 37° C. from a 3.8×10^{-4} M solution of silver nitrate containing Ag^{111} , by alcohol-fixed sections of rat spinal cord. The 'grand means' of a series of determination have been plotted. (Detailed results are given in table 1.)

In general, the value for the silver nuclei was greater after treatment with citrate buffer at pH 3.2, than with 2% sulphite. It seemed probable that the reducible silver was not completely removed by the citrate buffer. To determine the combination of silver with sections at low pH values, alcohol-fixed sections of rat spinal cord were impregnated at pH 3.2. These gave a total silver value of 11×10^{-4} g. Ag/ml. tissue and a silver nuclei value of 8.7×10^{-4} g. Ag/ml. tissue after treatment with citrate buffer at pH 3.2. Comparative figures for formol fixed human cerebrum were 4.4×10^{-4} g. Ag/ml. tissue and 2.5×10^{-4} g. Ag/ml. tissue.

(2) *The effect of concentration on silver uptake*

Alcohol-fixed sections of rat spinal cord were impregnated for 16 hours at 37° C. and pH 9 in solutions with silver nitrate concentrations of 3.8×10^{-4} M,

7.6×10^{-4} M, and 15.2×10^{-4} M. The total silver, developed silver, and silver nuclei were determined. The results are shown in table 2.

The uptake of each type of silver increased with an increase in the silver concentration in the impregnating bath, although the uptake was not proportional to the concentration in the impregnating solution. This confirms the results of the earlier staining experiments.

TABLE I

The effect of pH on the uptake of silver by alcohol-fixed rat spinal cord

pH value	Type of silver	Mean value g. Ag/ml. tissue $\times 10^{-4}$	No. of readings	Grand mean g. Ag/ml. tissue $\times 10^{-4}$
pH 9.0	Total	211	6	194
		176	5	
	Developed	114	7	107
		97	5	
	Nuclei (i)	30	6	35
	(ii)	41	5	
pH 8.0	Total	82	7	95
		95	5	
		113	5	
		119	3	
	Developed	72	7	70
		83	4	
		81	4	
	Nuclei (i)	17	5	21
	(ii)	26	4	
pH 7.0	Total	51	8	55
		62	4	
	Developed	37	6	41
		45	5	
	Nuclei (i)	19	6	17
	(ii)	14	4	
pH 5.6	Total	28	5	31
		27	3	
		37	4	
	Developed	12	5	17
		17	3	
		26	4	
	Nuclei (i)	6	6	9
	(ii)	16	3	

Nuclei (i)—treated with 2% Na_2SO_3 .

Nuclei (ii)—treated with citrate buffer at pH 3.2.

In this experiment, the value for the total silver was only 110×10^{-4} g. Ag/ml. tissue, as compared with a value of 194×10^{-4} g. Ag/ml. tissue obtained in the experiments on the effect of the pH value. Although alcohol-fixed sections of rat spinal cord were used in both experiments, the tissue was obtained from different animals. Such differences were found to occur throughout the experiments, even when the tissue was from the same block.

TABLE 2

The effect of concentration on silver uptake by alcohol-fixed sections of rat spinal cord

Concn. of silver in impregn. soln. ($\times 10^{-4}$ M)	Determination	g. Ag/ml. tissue $\times 10^{-4}$		
		Total Ag	Developed Ag	Ag nuclei
3.8	1	98.3	96.1	31.3
	2	128.5	86.4	32.4
	3	108.0	91.0	28.1
	4	108.0
	Mean value	110.7	91.2	30.6
7.6	1	131.8	130.7	38.9
	2	139.3	130.7	33.5
	3	150.1	124.2	34.6
	4	125.3	123.1	42.1
	Mean value	136.6	127.2	37.3
15.2	1	157.7	148.0	48.6
	2	150.1	130.7	40.0
	3	164.2	139.3	38.9
	4	152.3	143.6	40.0
	Mean value	156.1	140.4	41.9

TABLE 3

Uptake of silver by Nonidez-fixed rat spinal cord at different temperatures of impregnation

Temperature	Determination	g. Ag/ml. tissue $\times 10^{-4}$		
		Total Ag	Developed Ag	Ag nuclei
19° C.	1	78.8	31.6	13.5
	2	71.2	25.6	13.5
	3	63.7	27.0	12.4
	4	63.7	28.1	13.5
	Mean value	69.3	28.1	13.2
37° C.	1	71.3	55.7	16.8
	2	68.0	50.7	16.2
	3	73.4	50.7	14.3
	4	67.0	55.7	17.4
	Mean value	70.0	53.2	16.8

(3) *The effect of temperature on silver uptake*

Nonidez-fixed sections of rat spinal cord were impregnated in 3.8×10^{-4} M silver nitrate for 16 hours, at 19° C. and 37° C. respectively. The results for the total silver, developed silver, and silver nuclei are shown in table 3.

While there was little difference in the values for total silver, the silver reduced by the hydroquinone-sulphite developer increased by a factor of 2 from 19° C. to 37° C. This may be attributed to both a difference in the formation of silver nuclei at the two temperatures, and a relative difference in the temperature in the vicinity of the sections during development. However, it seems probable that the formation of silver nuclei is the most important

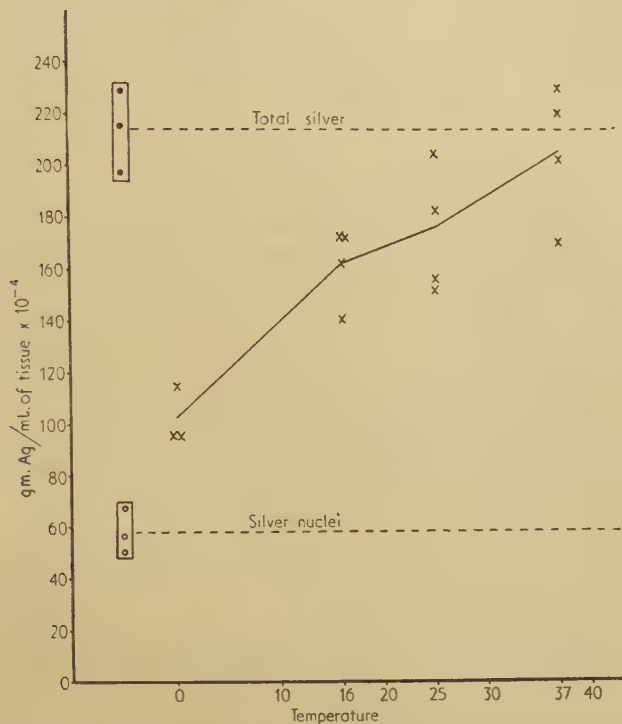


FIG. 2. The effect of the temperature of a 1% hydroquinone-10% sodium sulphite developing solution on the concentration of silver reduced by the developer. Total silver and silver nuclei values are shown. Formol-fixed sections of human cerebrum were impregnated in 3.8×10^{-4} M silver nitrate at pH 9 and 37° C.

factor, because in the previous experiments (Peters, 1955a), it was shown that development takes place most rapidly on the silver nuclei formed at higher temperatures. Furthermore, the sections were rinsed in distilled water previous to development, so that there would be little difference in the temperature of sections from the two solutions when they were immersed in the developer.

(4) The effect of temperature on the developing solution

Formol-fixed sections of human cerebrum were impregnated in 3.8×10^{-4} M silver nitrate at pH 9 and 37° C., before development in 1% hydroquinone-

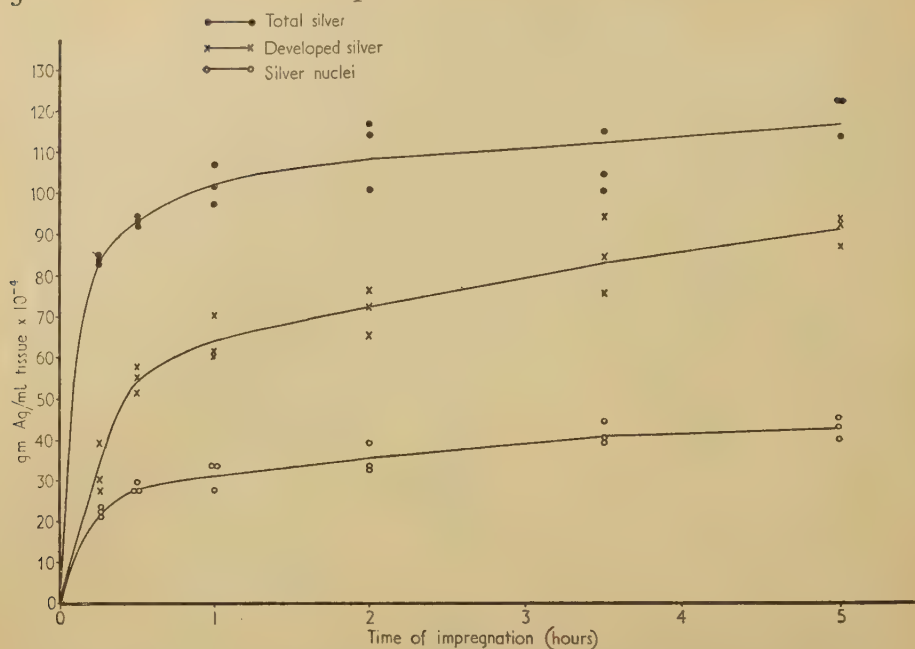


FIG. 3. The effect of time on the uptake of silver by formol-fixed sections of human cerebrum. Sections were impregnated in 3.8×10^{-4} M silver nitrate at pH 9 and 37°C .

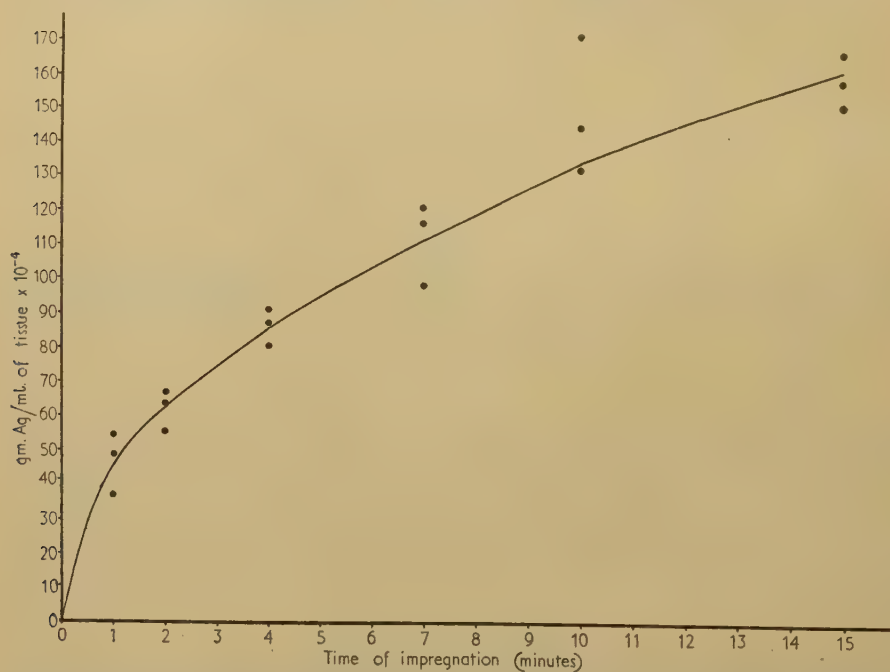


FIG. 4. The uptake of total silver by alcohol-fixed sections of rat spinal cord during the 15 minutes of impregnation. Sections were impregnated at 37°C . in 3.8×10^{-4} M silver nitrate at pH 9.

10% sodium sulphite at 0° C., 16° C., 25° C., and 37° C. The total silver and silver nuclei were determined. The results are shown in fig. 2.

The concentration of developed silver increased with an increase in the temperature of the developing solution, until at 37° C. there was an almost complete reduction of the reducible silver. At this temperature, the value for developed silver was 206×10^{-4} g. Ag/ml. tissue and that for total silver was 213×10^{-4} g. Ag/ml. tissue. This result could be appreciated visually. After development at 37° C., the staining was not so specific as that obtained at 25° C., so that a large portion of the developed silver at the higher temperature was accounted for by a staining of connective tissue.

(5) *The effect of time on silver uptake*

The effect of time on silver uptake was determined with a 3.8×10^{-4} M solution of silver nitrate at pH 9 and 37° C. Sections were removed from the impregnating solution at intervals and treated for total silver, developed silver, and silver nuclei. The results obtained with formol-fixed human cerebrum are shown in fig. 3. A similar type of uptake was obtained with Nonidez-fixed rat spinal cord.

In each case, there was a rapid initial uptake of reducible silver during the first 15 minutes. This was followed by a more gradual increase with time. The uptake of reducible or total silver during the first 15 minutes of impregnation was followed in more detail by using alcohol-fixed sections of rat spinal cord. The results are shown in fig. 4. This rapid initial uptake confirms the results of the staining experiments.

The rate of formation of silver nuclei followed a curve similar to that for the uptake of total silver. In the formol-fixed material, there was a rapid formation of silver nuclei, which may perhaps be attributed to the presence of free aldehyde groups derived from the fixative, but in the Nonidez-fixed material this phase of nucleus formation was less rapid. Furthermore, the silver nuclei form a larger portion of the total silver in the formol-fixed tissue than in the Nonidez-fixed tissue. Such a result was expected, because formol-fixed material is frequently light brown in colour when removed from the impregnating solution, while no reduction of silver is visible in Nonidez-fixed tissue.

The concentration of silver reduced by the developer follows a similar curve to that for the formation of silver nuclei. It was previously shown that the rate of development depends on the concentration of nuclei in the section (Peters, 1955a). The minimum time required to obtain a good staining is reflected by these curves. In formol-fixed material, a reasonably good depth of staining is obtained after about 1 hour of impregnation. With shorter times of impregnation, the staining is indistinct and lacking in detail. Any improvement in the depth of staining is characterized by an improvement in the detailed staining of the nerve-fibres. Under normal conditions of staining, such as those obtaining in the present experiments, the developed silver never attains a value higher than 70% of the total silver concentration.

(6) *The rate of deposition of silver by the glycine physical developer*

In an earlier paper (Peters, 1955b), a physical developing solution was described. This had the composition:

glycine	1.25 g.	} — 20 ml.
Na ₂ SO ₃ (anhyd.)	2.5 g.	
5% gelatine	25 ml.	
distilled water	225 ml.	
0.1 M citric acid / sodium citrate buffer at pH 6.3		— 20 ml.
1% silver nitrate solution		— 1 ml.

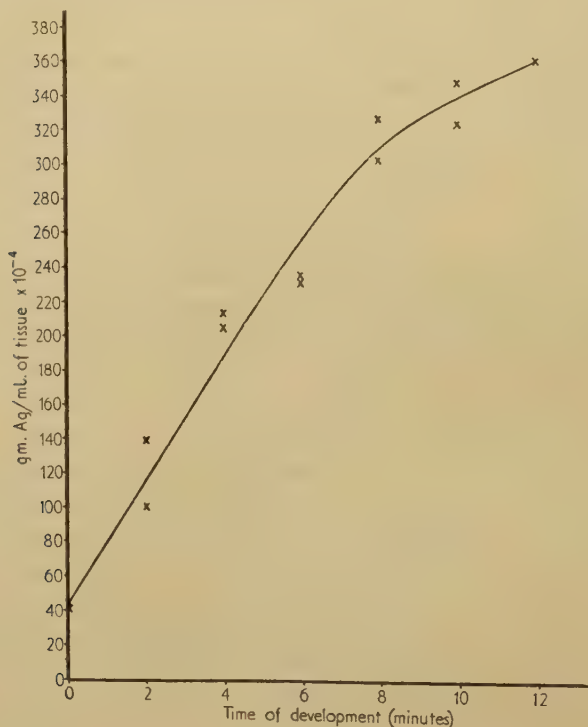


FIG. 5. The effect of time on the deposition of silver from the glycine physical developer. Formol-fixed sections of human cerebrum were impregnated at pH 9 and 37° C. in 3.8×10^{-4} M silver nitrate for 16 hours. The silver concentration at zero time of development is the value obtained for the concentration of silver nuclei in the sections.

To find the rate of deposition of developed silver from this solution on to silver nuclei, formol-fixed sections of human cerebrum were impregnated at pH 9 and 37° C. in 3.8×10^{-4} M silver nitrate for 16 hours, washed, and immersed in the citrate buffer for 1 hour to remove the reducible silver. The sections were washed in distilled water and transferred to a solution of the glycine physical developer at pH 6.3. Some sections were counted to determine the concentration of silver present in the sections as nuclei. In the developing

solution, radioactive silver nitrate was substituted for the 1 ml. of 1% silver nitrate in the above formula. Thus, during development, radioactive silver was deposited from the solution on to the sections. At intervals, sections were removed from the developing solution and transferred immediately to a 2% solution of sodium thiosulphate, which checked any further development. The sections were thoroughly washed before they were counted.

The results for the rate of deposition of silver from the developing solution are shown in fig. 5. The course of the deposition graph is consistent with the

TABLE 4
The uptake of silver by different rat tissues

Tissue	Determination	g. Ag/ml. tissue $\times 10^{-4}$		
		Total Ag	Developed Ag	Ag nuclei
Liver	1	216.0	224.6	71.3
	2	290.5	216.0	81.0
	3	304.6	166.3	89.6
	4	302.4	224.6	..
	Mean value	278.4	207.7	80.6
Heart-muscle	1	205.2	143.6	48.6
	2	183.6	169.6	36.9
	3	189.0	178.2	..
	4	..	169.9	..
	Mean value	192.6	165.2	42.8
Spinal cord	1	205.2	141.5	31.3
	2	158.7	130.7	23.8
	3	150.1	113.4	21.6
	4	..	122.0	..
	Mean value	171.3	126.9	25.6

assumption of a typical autocatalytic curve, as would be expected when further silver is reduced on to existing silver nuclei. Thus, the rate of deposition of silver increases with time. In this particular case, the rate of deposition began to fall off after about 8 minutes' development. At the end of 12 minutes' development, the sections were almost black in colour. This fall off in development was presumably due to the chemical reduction of silver in the developing solution itself. This would produce an effective decrease in the silver ions available for development of the sections.

(7) *The uptake of silver by other tissues*

Alcohol-fixed sections of rat heart-muscle, liver, and spinal cord were impregnated for 16 hours in 3.8×10^{-4} M silver nitrate at pH 9 and 37° C. The sections were treated for total silver, developed silver, and silver nuclei. The results are shown in table 4.

Both the heart-muscle and liver took up more silver than the nervous tissue. There was little visual difference in the general staining of the three types of

tissue, but the specific staining of the nervous elements was characteristically deeper than that of the other tissue elements.

DISCUSSION

In every case, the results of these quantitative experiments have confirmed the results of the earlier staining experiments (Peters, 1955*a*).

While the results for the total and developed silver must be valid, there is some reason to question the reliability of those obtained for the silver nuclei. These values were much higher than would be expected from the results of the staining experiments. At pH 9, for example, the nuclei were found to constitute 15% of the total silver and at pH 8, about 20%.

It was shown that some silver was retained by sections impregnated at pH 3.2 and afterwards treated with citrate buffer at the same pH value to determine the silver nuclei. Since it is unlikely that there is any formation of silver nuclei at this low pH, most of this silver must be in the form of combined silver which has not been removed by the citrate buffer. It is possible that a more correct value for the silver nuclei, determined in the experiments at other pH values after treatment with citrate buffer, would be obtained by subtracting from these figures for silver nuclei the amount present after impregnation at pH 3.2. However, since there are no experimental data to support this view, no corrections have been made in the presentation of the results for the concentration of silver present as nuclei.

The values obtained with sodium sulphite treatment were lower than those obtained with the citrate buffer, and this in itself suggests that the citrate buffer values were too high. Presumably the action of the sulphite is to complex the reducible silver; since the sulphite-silver complex is more stable than the histidine-silver complex, silver ions would be removed from the tissue. Such a reaction would also be accompanied by a formation of silver sulphide, which might contribute towards an over-estimate of the values of silver nuclei.

The uptake of silver with time throws more light upon the silver nuclei values. From these results (fig. 3), it will be seen that the formation of silver nuclei follows the uptake of total silver closely. However, the visible staining depth indicated that there was a much slower increase in the formation of nuclei, since tissues from fixatives other than formol showed no appreciable staining at least for the first hour of impregnation. From the present quantitative experiments, the concentration of nuclei after 20 minutes' and 1 hour's impregnation were almost the same, so that the same density of staining would be expected at both times. This result was not found. Therefore, it is probable that a greater part of the value for the silver nuclei concentration must be attributed to reducible silver which was not removed by either the citrate buffer or the sodium sulphite.

Before any further quantitative work on the formation of silver nuclei can be carried out, a method must be found which will ensure a complete removal of the reducible silver.

The experiments of the effect of time on the combination of reducible silver with the sections (figs. 3 and 4), confirm the earlier result that the combination is complete within 15 minutes of the commencement of impregnation. Thus, the longer period of impregnation which is necessary before good staining is obtained, must be due to the slower rate of formation of silver nuclei.

The figures for the concentration of developed silver obtained for the frog spinal cord by the use of isotopes, agrees closely with those obtained in the

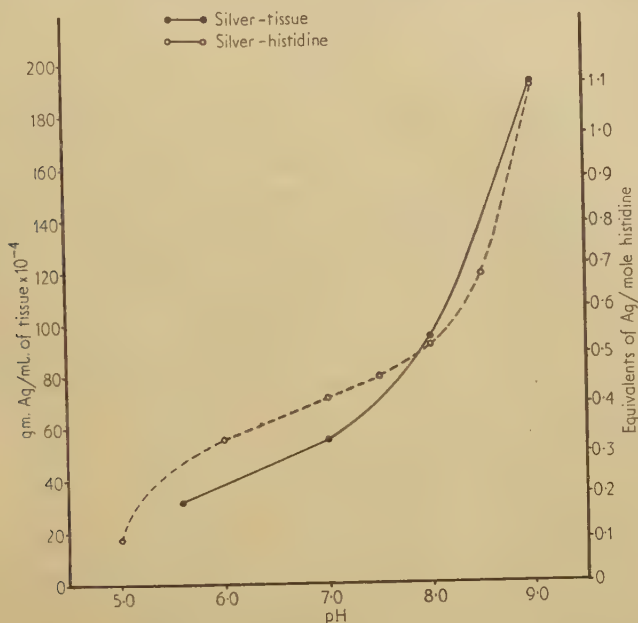


FIG. 6. Comparison of the effect of pH on the combination of total silver by nervous tissue (full line and left-hand vertical scale) and the effect of pH on the combination of silver with histidine (broken line and right-hand vertical scale). The silver histidine curve was obtained from the results of Haarmann and Frühauf-Heilmann (1941).

initial experiments when an adaptation of the Wigglesworth microtitration method for chloride (1937) was employed. The value for developed silver by the titration method was 171×10^{-4} g. Ag./ml. tissue, compared with 199×10^{-4} g. Ag./ml. tissue obtained by the isotopic method.

The results for the total silver uptake at pH 9 show that the sections concentrate the silver from the impregnating bath by about 500 times. Thus, there is a definite uptake of silver by the sections.

Hill and Branch (1940) showed that the imidazole ring of histidine has a pK_b value of 7.97 with respect to salt formation. The type of curve obtained for the effect of pH on the combination of total silver is similar to that which would be expected from an ionizing group with a pK value of about 8.

Haarmann and Frühauf-Heilmann (1941) determined the equivalents of silver combined with histidine and showed that at pH 7 only 0.41 equivalents

of silver combine with 1 mole of histidine, while the number increased to 1.12 at pH 9. Their figures for the combination have been superimposed on the graph for the effect of pH on the uptake of total silver obtained in the present experiments (fig. 6.) The trend of the two sets of results is very similar and is further evidence for the theory that the majority of the silver is combined with histidine during impregnation.

Haarmann and Frühauf-Heilmann also showed that at pH 10.0, 2.02 equivalents of silver were bound with histidine. Therefore, they postulated that the silver is bound to both the imidazole ring and the primary amino-group in the side chain.

Block (1945), on the basis of a 16% nitrogen content, has shown that the concentration of histidine in the mammalian brain is 2.6% by weight. Assuming the density of the tissue to be unity, this is equivalent to a concentration of 0.16 M. On the basis of the same assumptions, at pH 9 the concentration of total silver would be 0.19 M for the rat spinal cord and 0.13 M for the human cerebral material. Thus, these data are consistent with the combination of silver with histidine in the tissue.

Temperature has little effect on the combination of reducible silver with tissue (table 3); its main effect is to increase the formation of silver nuclei. This is indicated from the results, but it also appears probable that a more reliable index of nucleation is the value for developed silver formed after impregnation at the two temperatures.

The effect of change in concentration of silver on the combination is not very great, although there is some increase in the total silver in the section, with an increase in the concentration in the impregnating solution. Since the tissue is not saturated with silver at all concentrations in the outside medium, the silver complex must exhibit a reversible combination.

In some ways, the choice of rat heart-muscle and liver to determine the silver uptake by other tissues was unfortunate, because both tissues have a high haemoglobin content. It was shown previously (Peters, 1955a) that silver combines extensively with haemoglobin as a consequence of its high histidine content. A further factor contributing to the high total silver concentrations in heart-muscle and liver, is the density of the fixed tissues, for both have a lower water content than nervous tissue. Nevertheless, this experiment shows clearly that combination with silver during impregnation is not a reaction which is specific to nervous tissue. It appears also that the silver nuclei themselves are not confined to the nervous elements, so that the specificity of the final stain is determined during development.

To summarize, these experiments show that there is a considerable concentration of silver by sections during impregnation, and the results have quantitatively confirmed the earlier staining experiments. These quantitative results appear to be in every way consistent with the hypothesis that the reducible silver combines with the imidazole ring of histidine and have provided further evidence for the assumption that the final staining is determined during development, and not during impregnation.

I wish to express sincere thanks to Professor J. E. Harris for his interest and advice during the course of this work, which was carried out during the tenure of a maintenance grant from the Department of Industrial and Scientific Research. I have also to acknowledge the use of a Geiger counter provided for the Department by the Medical Research Council.

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Experiments on the Mechanism of Silver Staining

IV. Electron Microscope Studies

By A. PETERS

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With one plate (fig. 1)

SUMMARY

Silver-stained sections of nervous tissue were examined with an electron microscope. It has been shown that the developed silver is particulate. These particles are between 30–700 Å in diameter and in nerve-fibres they are disposed along the surface and inside the components of the fibre. When impregnated sections were developed in a glycine physical developer, the mean size of the developed silver particles increased with an increase in the time of development.

In silver-stained sections of human sympathetic ganglion, the silver particles were distributed throughout the cell, but were more densely aggregated in the cell membrane and the nucleus. Erythrocytes show a dense aggregation of silver particles throughout the cytoplasm.

INTRODUCTION

IN previous studies (Peters, 1955, *a* and *b*), it was shown that during the impregnation stage of silver staining, silver is taken up by the sections from the impregnating solution. This silver consists of two distinct fractions. Most of the silver is in an unreduced state which is in combination with histidine and other amino acids, while a smaller fraction consists of nuclei of reduced silver. When the impregnated sections are immersed in a developing solution, such as hydroquinone-sulphite, the combined (reducible) silver is reduced by the developer and deposited on to the silver nuclei.

After development, the light microscope shows the silvered regions of the sections to be homogeneous, i.e. there is no apparent differentiation of the silver into particles, as would be expected from the role of silver nuclei in development. Therefore, to investigate the state of the developed silver in the sections, an electron microscope examination was carried out on the silver-stained sections.

METHOD

Blocks of nervous tissue were fixed and embedded in paraffin wax; sections were cut at 25 μ . The sections were not mounted on to slides, but dewaxed, taken down to distilled water, and then stained while floating free in the solutions. Impregnation was carried out for 16 hours at pH 9 and 37° C. in 1/20,000 silver nitrate. Sections were developed either in a developer of 1% hydroquinone in 10% sodium sulphite or in a glycine-containing developer (Peters, 1955*b*). They were then washed in running tap-water followed by distilled water, and taken up to absolute alcohol before being transferred to several changes of an unpolymerized plastic embedding material.

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The material used to re-embed the sections, before they were cut for examination under the electron microscope, was 90% methyl methacrylate (supplied as 'Kallodoc' by I.C.I.) with 10% of tricresyl phosphate as a softener. The 'inhibitor' was removed from the Kallodoc by washing the liquid several times in 5% sodium hydroxide, until the hydroxide fraction was no longer brown. The plastic was then thoroughly washed with distilled water to remove all the sodium hydroxide and dried over calcium chloride. The above formula for the plastic was obtained after several trials in which various concentrations of tricresyl phosphate were added to the Kallodoc. The plastic was polymerized in front of an ultra-violet lamp.

To embed the silver-stained sections, plastic was poured into the bottom of small gelatin capsules to a depth of about 4 mm. and then polymerized. When this layer had hardened, a second layer of unpolymerized plastic was poured on to the top. The sections were transferred to this unpolymerized layer. To make the sections more compact, a wedge of polymerized plastic was lowered on to them and the gelatin capsule again placed in front of the ultra-violet lamp until the plastic was polymerized throughout. No demarcation was visible between the various layers of plastic after this final hardening, but the sections were visible as a thin compact area in the block.

Sections for examination under the electron microscope were cut at room temperature on a Cambridge rocking microtome with an advancing mechanism of 0.1μ . A glass knife was used and the sections were floated off on to a water-bath fixed to the knife. From the water-bath, the sections were lifted off on to copper grids on which had already been mounted a supporting layer of formvar.

After drying, the sections were examined with a Phillips electron microscope. No attempt was made to remove the plastic from the sections.

RESULTS

Examination of the sections (fig. 1, A-F) shows that the silver produced by development is particulate. It can be seen from the photographs that the particles of silver are not deposited in the interstices between the nerve-fibre

FIG. 1. Electron micrographs. All material impregnated in 1/20,000 silver nitrate at pH 9 and 37° C. (A, axon; C, cytoplasm; E, erythrocyte; M, myelin sheath; N, nucleus.)

A, formol-fixed human sympathetic ganglion. Developed in glycine physical developer. Note distribution of particles of developed silver in the nerve-cell and in the erythrocytes.

B, longitudinal section of nerve-fibre from alcohol-fixed frog sciatic nerve. Developed in glycine physical developer. Developed silver particles orientated along the surface and inside the fibrils of the nerve-fibre and particles in the myelin sheath.

C, cell from formol-fixed human sympathetic ganglion. Developed in glycine physical developer. Note distribution of particles in the nucleus and along the cell membrane.

D, longitudinal section of nerve-fibre from alcohol-fixed frog sciatic nerve. Developed in hydroquinone-sulphite. Developed silver particles along the surface and inside the fibrils of the nerve-fibre.

E, longitudinal section of formol-fixed rat sciatic nerve. Developed in glycine physical developer for 15 minutes. Compare with F and see fig. 2, A. Note the developed particles of silver in the myelin sheath.

F, transverse section of formol-fixed rat sciatic nerve. Developed in glycine physical developer for 1-2 minutes. Compare with E and see fig. 2, A.



FIG. 1
A. PETERS

components, but along the surface of and inside these components (fig. 1, B, D, and E).

The size of the silver particles and their positions in the fibres are similar irrespective of whether the nerves have been fixed in alcohol, formol, or

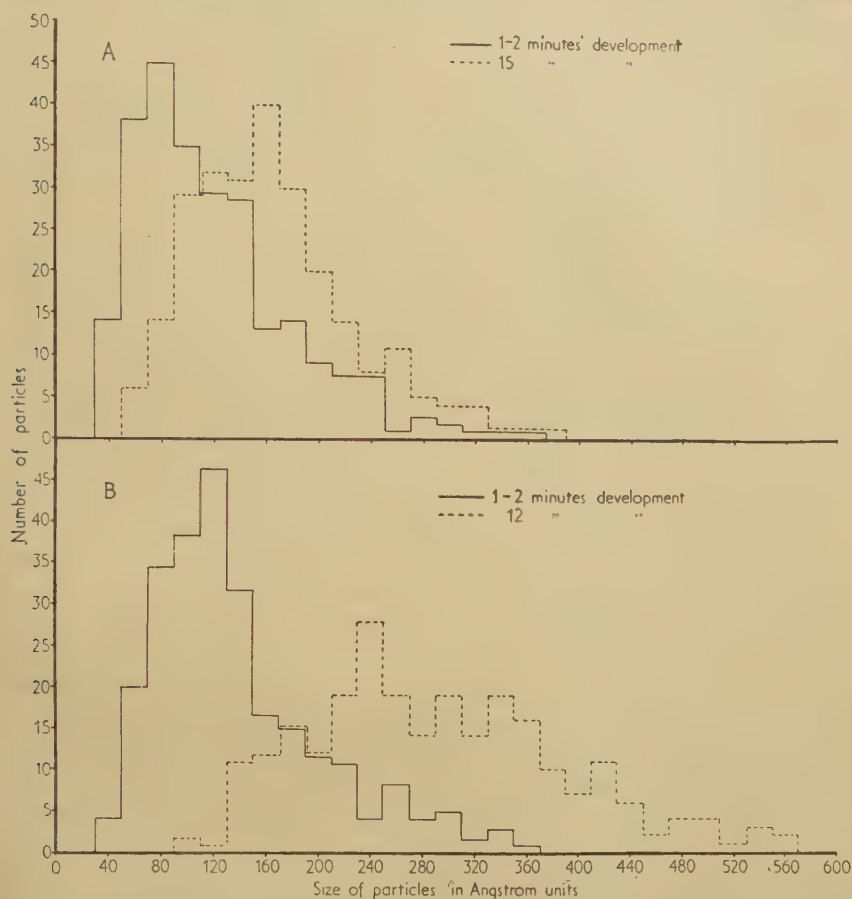


FIG. 2. A comparison of the ranges of sizes of developed silver particles, obtained after different times of development. Sections of frog sciatic nerve were developed in the glycine physical developer. The sizes of 250 particles are plotted for each time of development. Particle sizes were measured from electron micrographs. A, formol-fixed sections: 1-2 minutes' development and 15 minutes' development. B, alcohol-fixed sections: 1-2 minutes' development and 12 minutes' development.

chloral hydrate (Nonidez). These fixatives have been used throughout this investigation, so that the characteristics of the silver particles were determined in sections that had been stained by the normal staining procedures.

The effect of time of development on the size of the developed silver particles were determined by the use of a glycine physical developer (Peters, 1955b). In these experiments the sections were impregnated and treated with 2% sodium sulphite to remove the reducible silver previous to development

(Samuel, 1953). In the first experiment, one batch of formol-fixed sections of frog sciatic nerve was developed for 1–2 minutes and another for 15 minutes. Typical results are shown in fig. 1, E and F. It will be seen that the particle size increases with an increase in the development time. Plots of diameters of 250 particles measured at random from the photomicrographs of each batch of sections are shown in fig. 2, A. With 1–2 minutes' development, the size-peak of the particles was 50–110 Å and after 15 minutes' development it was 90–180 Å. In the second experiment, alcohol-fixed sections were developed for 1–2 minutes and 12 minutes. Here the peak range of diameters for 1–2 minutes' development was 50–130 Å and after 12 minutes' development it was 210–350 Å. The results are shown in fig. 2, B.

Sections were examined for silver nuclei. These sections were impregnated in the usual way and treated with citrate buffer at pH 3.2 to remove the reducible silver (Peters, 1955*b*). Development was omitted. In no case were any silver particles visible under the electron microscope, even when formol-fixed material was examined. The formol-fixed material showed a light brown colour, due to the reduction of silver, on removal from the impregnating bath.

The distribution of silver particles in cells was investigated in sections of formol-fixed, human sympathetic ganglion. This material was chosen because of the large number of nerve-cell bodies which are present. An earlier examination was carried out with sections of spinal cord and brain tissue, but owing to the relative sparsity of cells in such tissues, nothing that could be definitely identified as cell-body was visible after re-sectioning.

In the sympathetic ganglion material, the silver particles were distributed throughout the cell-bodies (fig. 1, A and C).

In all cases there was a dense aggregation of particles on the cell membrane but not on the nuclear membrane. A typical nerve-cell is shown in fig. 1, A, and this shows the distribution of silver particles clearly. In the same figure are four red blood-cells and in these the silver is distributed evenly, but rather densely throughout the cells, as would be expected from the dark staining of such cells and the affinity of haemoglobin for silver (Peters, 1955*a*). In all cells the nucleus contained more particles per unit volume than the cytoplasm, which would account for the deep staining of the nucleus.

DISCUSSION

This investigation has shown that the developed silver is particulate. The particles are generally spherical, with a diameter of 30 Å to 700 Å. Assuming the radius of the silver atom to be 1.26 Å (Glasstone, 1946, p. 383), then these particles contain between 1.7×10^3 and 20×10^6 silver atoms. No account has been taken of the packing fraction of the silver atoms.

In the previous electron microscope studies made by Baud (1951 and 1952) and Baud, Baumann, and Weber (1951), material fixed by osmium tetroxide was stained by the method of Weber (1947). These workers concluded that by this method of staining the silver was deposited in the spaces in between

the protofibrils of the fixed tissue. Baud (1951) found that these protofibrils were in the form of homogeneous filaments which were 200–300 Å long and disposed both parallel to each other and to the long axis of the nerve-fibre. Thus, regular spaces would arise and it was in these that Baud found the silver particles arranged in a linear series. The rows of particles were orientated along the long axis of the fibre, so that the size and number of spaces were supposed to determine the dichroic property of the silver stain. Dichroism can be observed after staining by the method of Weber (see Baud, 1948). While there is no doubt that dichroism can occur after silver staining, the reproductions of the electron micrographs produced by Baud (1951) and Baud, Baumann, and Weber (1951) to show the arrangement of silver particles are not very convincing.

Frey-Wyssling and Walchi (1946) have studied the dichroism obtained after impregnating cellulose fibres with silver and state that it can be explained on the basis of the formation of metallic silver particles in the submicroscopic spaces of the fibres. X-ray data quoted by these authors showed that the silver was adsorbed in the form of non-orientated cubic silver. No dichroism could be detected in the sections of nerve-fibres stained by the present method, and the micrographs show that the silver was deposited in and on the fibre components, but not in the interspaces.

In fig. 1, B and D, the fixation can be seen to have produced definite submicroscopic fibrils inside the nerve-fibre. It is possible that deposition of silver along such fibrils gives rise to the classical neurofibrils when such sections are examined under the light microscope. A similar explanation was put forward by Baud (1951), who examined the silver particles deposited in the brain of the mouse.

It can be seen from fig. 1, E and F, that the number of visible silver particles increased with an increase in the time of development in the physical developer. This may be due either to the fact that the smaller particles were not visible under the electron microscope, or that not all of the silver nuclei act as centres of development. In the first case, the increase in the depth of staining which is obtained by increased development, would be explained by an increase in the particle size alone. In the second case, there may be an increase in the number of developed silver particles, but whether this actually takes place could not be determined. No measurements were made of the numbers of silver particles per unit volume after different times of development, since there were appreciable variations in the thickness of the sections.

One probable explanation for the fact that no silver nuclei could be detected is that the size of the silver nuclei was below the limits of resolution of the electron microscope. The number of silver atoms necessary to form a centre of development is very small (only 3 or 4 silver atoms are necessary to form a centre of development in an exposed photographic emulsion (Mitchell, 1954)).

It can be seen from the photographs (fig. 1, B and E), that the silver particles are not confined to the nerve-axon itself, since small particles are visible in the

myelin sheath. This lack of specificity would be expected from the previous experiments in development, because it was shown that only a relatively small number of developing agents produce a specific stain (Peters, 1955*b*). However, the particles of silver in the myelin sheath tend to be smaller and fewer in number than those in the axis cylinder, so that they would play only a small part in the formation of the image which is visible under the light microscope.

Thus, after staining by the present method, the particles of developed silver are deposited in and on to the fibrils in the nerve-fibre, but not in the interspaces. In the sections of sympathetic ganglion the particles were distributed throughout the cell-bodies, but were more dense at the cell-membrane and in the cell-nucleus. A fairly dense aggregation of silver was also found in the red cells. In general, the regions of the cell which are darkly stained under the light microscope are the regions of the section which contain the greatest number of silver particles per unit volume.

I wish to express sincere thanks to Professor J. E. Harris for his interest and advice during the course of this work. I am indebted to Professor T. Hewer of the Pathology Department for the loan of the special microtome and to Dr. A. Lee for early help with the sectioning technique. Assistance with the electron microscope was given by technicians of the Physics Department. This work was carried out during the tenure of a maintenance grant from the Department of Scientific and Industrial Research.

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A General-Purpose Method of Silver Staining

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SUMMARY

A method of silver staining for paraffin sections has been described. Sections should be fixed in either Nonidez fixative, 4% formaldehyde, or 4% formaldehyde saturated with mercuric chloride. The sections are impregnated for 16 hours in 1/20,000 silver nitrate at pH 8 or 9 and developed in a glycine physical developer after the reducible silver has been removed with a 2% solution of sodium sulphite.

The effect of pH on impregnation has been described. A spectrum of staining was obtained in which nerve fibres began to stain appreciably at pH 7, cell nuclei at pH 8, cell cytoplasm at pH 9, and connective tissue at higher pH values. Therefore, impregnation should be carried out at pH 8 to obtain a good staining of nerve fibres and at pH 9 if some staining of cell bodies is also required.

IN recent years, a number of methods for the silver staining of paraffin sections of nervous tissue have been described. Probably the most important have been those of Holmes (1947), Romanes (1950), and Samuel (1953*b*). Although these methods vary in detail, they have the common factor that impregnation is carried out at a controlled pH in a dilute solution of a silver salt. Holmes and Samuel used silver nitrate and Romanes used silver chloride. While Holmes and Romanes employed a hydroquinone-sulphite developer, which reduced the silver taken up by the sections during impregnation, Samuel removed the reducible silver with a sodium sulphite solution and developed in a physical developer. Thus, in Samuel's method the silver which is reduced to produce the final staining picture is derived from the developing solution and not from the silver combined with the section during impregnation (see Peters, 1955*b*). By this means the development process can be controlled to a greater extent than is possible with the chemical developers, such as hydroquinone-sulphite.

The staining method to be described in this paper has been evolved as a result of a series of experiments on the mechanism of silver staining (Peters, 1955 *a*, *b*, *c*, and *d*). Some observations on fixation and the effect of pH of the impregnating solution will also be considered, because these two factors play an important part in the production of the final staining picture.

Fixation

Rowe and Hill (1948) considered the use of various fixatives before staining by the method of Holmes (1947). They concluded that Susa, chloral hydrate, and mercury-formol fixatives gave the best results, but they pointed out that chloral hydrate fixatives produce severe shrinkage of the tissue.

A series of simple fixing agents, including picric acid, potassium dichromate, chromic acid, 70% alcohol, and formalin were tested. The results showed

that of this group, only formalin- and alcohol-fixed tissue gave consistently good staining results. However, while alcohol-fixed material gave rise to a good staining picture, the fixation was poor. As a result of these and other experiments, it was concluded that the best staining was produced after fixation in Nonidez fixative (25 g. of chloral hydrate in 100 ml. of 50% alcohol (Nonidez, 1939)) in 4% formaldehyde, and in 4% formaldehyde saturated with mercuric chloride.

While the Nonidez fixative produced some shrinkage of the tissue, it had the advantage that, after staining, the nerve elements stood out clearly against the other tissue elements. Formalin produced better fixation than the chloral hydrate, but the staining of the nerve fibres was not so well differentiated. The addition of mercuric chloride to the formalin has the effect of increasing the depth of staining of the nerve fibres and suppressing the staining of the background, so that the contrast was improved. When mercury-formol is used, the precipitate which is formed during fixation must be removed from the tissue with 2% iodine in 70% alcohol before impregnation in the silver solution. The excess iodine should not be removed from the sections with sodium thiosulphate, because, unless the thiosulphate is completely removed, it interferes with the silver staining.

The pH of the impregnating solution

The effect of pH on the staining of the cerebellar region of the rat's brain was determined over the pH range 4.5 to 11.2. Sections were impregnated in a 1/20,000 solution of a silver salt for 16 hours at 37° C. A solution of silver nitrate was buffered at pH 4.5 and 5.6 with sodium acetate / acetic acid buffer and at pH 7.0, 8.0, and 9.0 with borax / boric acid buffer. At pH 9.4, 9.9, 10.3, 10.7, and 11.2 a 1/20,000 solution of the silver diammine complex was employed and the pH of the solution was controlled by the addition of either sodium carbonate or ammonia. (Silver nitrate could not be used at these high pH values because of the formation of silver hydroxide.) The sections were either developed in 1% hydroquinone / 10% sodium sulphite or in the glycine physical developer.

After development in the hydroquinone-sulphite developer, there was an increase in the density of staining from pH 4.5 to 9.0. When the pH was controlled by the addition of sodium carbonate to the diammine complex, staining increased from pH 9.4 to 11.2, but when the pH was adjusted with ammonia, there was a fall-off in the intensity of staining with an increase in the pH. The addition of ammonia probably reduced the ionization of the diammine complex and thereby suppressed the release of free silver ions available for staining. Thus, the solution was stabilized, and any tendency for it to reduce or combine with proteins of the nervous tissue was retarded (Peters, 1955a). At higher pH, when the greatest concentration of ammonia was added to the solution, there was the lowest concentration of silver ions available for staining.

As long as the free silver ion concentration in the impregnating solution was

constant, the intensity of staining, on development with hydroquinone-sulphite, increased with the pH. As the pH was raised, there was a tendency for the deposited silver to become coarse, although this factor was not important until about pH 10·7.

Development with the glycine physical developer showed that with a constant silver ion concentration in the impregnating solution, the formation of

TABLE I
The effect of pH on silver staining

pH	Intensity of staining				Comments
	Nerve fibres	Nuclei	Cytoplasm	Connective tissue	
4·5-5·6	+	+	+	+	Staining too light to show details.
7·0	++	+	+	+	Fibres begin to stain. Staining still very light.
8·0	+++	++	+	+	Fibres contrasted against light background.
9·0-9·9	++	+++	++	+	Quite good staining of all nerve elements.
10·3	++	++	++	++	Fibre staining rather coarse.
10·7	+	+	+++	++	Cell nuclei appear as outlines. Staining generally coarse.
11·2	+	+	+++	+++	Few details visible; staining very coarse and homogeneous.

+ faint; ++ distinct; +++ deep.

silver nuclei increased with an increase in the pH value of impregnation (see Peters, 1955a).

The effect of pH on the staining of the various nerve elements and connective tissue is shown in table 1. An interesting point brought out by this table is the spectrum of nerve-element staining which is obtained as the pH is raised (Silver, 1942). Thus, fibres and nuclei begin to stain at the lower pH values, while the cytoplasm only begins to stain appreciably at pH 9·0. Moreover, the specific staining of the cytoplasm persists to a higher pH than that of the nerve fibres and cell nuclei. Some of the specificity is lost at higher pH as a result of a staining of the connective tissue.

It can be seen from table 1 that the best pH for impregnation is 8·0 to 9·0, because over this range the deepest staining of the nerve fibres and nuclei is obtained. In general, the staining of the nerve fibres is deepest at pH 8·0 and there is little staining of the other tissue elements at this pH. At pH 9·0 the staining of the cell nuclei is deeper, but there is some reduction in the intensity of staining of nerve fibres. However, a more complete general

staining of the nerve elements results at pH 9.0 than at pH 8.0. At all other pH values the staining is either too light or too unspecific.

In their methods, Holmes (1947) impregnated at pH 8.5, Romanes (1950) at pH 9.0, and Samuel (1953*b*) at pH 6.78.

A possible explanation for the spectrum of staining lies in the physical state of the proteins at the different pH values. Silver (1942) suggested that the spectrum effect was caused by a variation in charge on the cellular components, so that parts of the cell stain at different pH values and have an 'optimum magnitude of charge to absorb the nascent colloidal silver'. Although it is doubtful if Silver's theory of staining is generally correct (see Holmes, 1947, and Samuel, 1953*a*), the pH effect may well be due to a difference in charge on the proteins at different pH values. A further possibility is that the sites of formation of the silver nuclei change with pH; this would lead to the developed silver being deposited at different sites over the pH range. Such a change in the sites of formation of nuclei could be attributed to a change in the redox potential of the cell proteins with the pH value (see Peters, 1955*a*). Thus, the number of silver nuclei formed at a particular site would depend on the value of the redox potential at that site.

Method of staining

(1) Fix blocks of tissue in either Nonidez fixative (1939) or 4% formaldehyde or 4% formaldehyde saturated with mercuric chloride.

(2) Mount paraffin sections on slides with albumen, dewax, and take to water. (If mercury-formalin has been used for fixation, remove the precipitate from the sections with 2% iodine in 70% alcohol.)

(3) Impregnate sections in the following solution in an incubator for 16 hours:

1 ml. of 1% silver nitrate, 180 ml. of distilled water, and 20 ml. of 0.1 M boric acid / borax buffer at pH 8 or 9. The standard buffer solution is made by mixing solutions of 0.1 M boric acid and 0.1 M borax until the required pH, as indicated by a glass electrode, is attained.

Impregnate at 37° C. for material fixed in chloral hydrate and at 56° C. or 37° C. for formalin and mercury-formalin material.

(4) Transfer sections to 2% sodium sulphite for 5 minutes to remove the reducible silver (Samuel, 1953*a*).

(5) Wash in several changes of distilled water.

(6) Develop the sections in the following glycine-containing physical developer until the required depth of staining is attained. Sections should be examined at intervals during development. The usual time is 2–5 minutes.

Stock solution:	glycine	1.25 g.	} 20 ml.
	Na ₂ SO ₃ (anhyd.)	2.5 g.	
	5% gelatine (powdered B.P.)	25 ml.	
	distilled water	225 ml.	

0.1 M citric acid / sodium citrate buffer at pH 6.3 20 ml.

1% silver nitrate solution 1 ml.

- (7) Wash in running tap water for 10 minutes.
- (8) Dehydrate, take through absolute alcohol to xylene, and mount in Canada balsam.

The stock solution of the developer is quite stable. To prepare this the glycine and sodium sulphite are dissolved, by warming, in about 100 ml. of distilled water and the warm gelatine solution is added immediately. The volume is then made up to 250 ml. The optimum pH for development may vary slightly with the sample of gelatine; initial tests should be carried out with citrate buffers over the range pH 6.0 to 6.5. However, once the pH value has been determined for any particular sample of gelatine, no further tests are necessary. The citrate buffer controls the pH value at the site of development; on either side of the optimum pH the silver deposition is rather coarse.

In general, no toning is necessary, because the fibres show up black against a green background and therefore give a good contrast.

The reducible silver may be removed with a citrate buffer at pH 3.2, but the sodium sulphite is more convenient to prepare. Unless the reducible silver is removed, the initial development is rapid and tends to be somewhat un-specific.

The temperature of impregnation varies with the fixative and the type of tissue. For example, formol-fixed rat cerebellum produced the best staining picture at 56° C. and formol-fixed human cerebellum at 37° C. On the other hand, material fixed with chloral hydrate never produced specific staining after impregnation at 56° C.

Whether the sections are impregnated at pH 8 or 9 depends on the nerve elements that are required in the final staining picture. As has already been pointed out, at pH 8 the staining of the nerve fibres is deep and that of the non-nervous elements is light, while at pH 9, although more background is stained, the nerve-cell bodies stain more intensely.

Deeper staining may be produced by treating the sections with 20% silver nitrate for 1 hour before impregnation (Holmes, 1947). In the present method this step is generally unnecessary.

It is recommended that embryonic tissues should be fixed in Nonidez fixative and dehydrated in the Lang series of alcohols (Lang, 1937).

A less perfect staining of nerve fibres may be achieved by developing the impregnated sections in one of the following solutions. Here, development follows step 3 in the above scheme.

- | | |
|---|---------|
| (1) hydroquinone | 1 g. |
| Na ₂ SO ₃ (anhydrous) | 10 g. |
| distilled water | 100 ml. |
| Warm the solution to 20° C. before use. | |
| (2) chloroquinol | 1 g. |
| Na ₂ SO ₃ (anhydrous) | 4 g. |
| distilled water | 100 ml. |

Use at room temperature.

While the basic method of staining in the above scheme produces good results, the modifications have been listed because a rigid method of staining cannot be expected to produce the best possible results with all tissues and fixatives. Thus, initial trials should be carried out to determine the pH and temperature of impregnation which gives the best staining with the sections available.

This method has been used successfully with fish, amphibian, and mammalian tissues, including brain, spinal cord, sciatic nerve, sympathetic ganglia, muscle end-plates, and embryonic material.

Note.—The glycine mentioned in these papers is the compound called by that name in photography; that is to say, *p*-hydroxyphenylglycine.

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Studies of the Impregnation of Nervous Tissue Elements

I. Impregnation of Axons and Myelin

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SUMMARY

All metallic impregnation methods depend on the presence of reducing groups in tissues. By testing the effect of various reduction indicators on sections of nervous tissue it was found that some reduction systems do not have a high enough oxidizing potential to serve for the visualization of either axons or myelin. Others can visualize myelin sheaths. The systems having the highest potential visualize also axons. Although myelin can be oxidized more easily than axons, axons are probably relatively richer in reducing groups.

Impregnation by AgNO_3 or Ag-diammine results in the formation of silver nuclei in both axons and myelin. Subsequent exposure of such sections to the action of reducing agents increases the visible impregnation of the axons much more than that of the myelin. This effect can be ascribed to the low dielectric constant of myelin.

Gold toning serves a dual purpose: (a) coating with gold the more heavily impregnated structures, and (b) clearing the background through the displacement of the silver from the less intensely impregnated areas.

The mechanism of metallic impregnations and the rationale of the different steps in Bielschowsky's procedure for axons are discussed.

INTRODUCTION

ALTHOUGH metallic impregnation methods are of the utmost importance in the study of normal and pathological nervous tissue constituents, they are still universally considered to be most capricious. For no understandable reason they sometimes succeed and at other times fail. Furthermore, the chemical basis of these methods is not clearly understood, and their practical application represents more often an art than a science. The possible pitfalls in studies which are based on specific impregnations are numerous, as a slight change in technique, which may pass unnoticed, might result in the staining of structures other than those for which the method is considered to be selective.

The present series of studies aims at showing that all the metallic impregnation methods are based on a single general principle which can be deduced from the available information. According to this principle all metallic impregnations depend on the reducing capacity (redox potential) of the different structures, an idea expressed although not substantiated by Palmgren (1948). The various structures differ from each other and allow differential staining because of the following: (1) The reducing groups may be either free, or may

be formed, or made available, by different preliminary treatments. (2) The redox potential of the structures may be different. (3) The rate of reaction of the chemical reducing groups with different reagents may be different. (4) The amount of reducing activity (number of reducing groups per unit of volume) may vary in the different structures. (5) Treatment subsequent to the reduction of the metal may effect differently various structures.

The experiments reported in this paper were performed in order to clarify the mechanism of the metallic impregnation of axons and myelin. The mechanism of impregnation of other nervous tissue elements will be dealt with in subsequent papers.

MATERIAL AND METHODS

Most experiments were performed on human autopsy material fixed within less than 8 hours after death. In some experiments dog and cat material was used for comparison. Pieces of cerebral cortex and of medulla or spinal cord were used, and their sections were usually processed simultaneously. Tissue sections were prepared as follows:

(a) *Frozen sections.* Preliminary experiments showed that the floating of sections, or parts of them, on the surface of the impregnating solution, and folding of the sections introduced disturbing effects and artifacts. These effects were probably due in the first case to the disturbing effect of atmospheric oxygen on oxidation of tissue elements by the metal ions, and, in the case of folding, to the different speed of penetration of reagents into the sections. Frozen sections cut at 15–20 μ were therefore affixed to albuminized slides according to the method of Iwanoff (cf. Roulet, 1948) in accordance with Feyrter's (1951) suggestion.

(b) *Carbowax-embedded sections.* Further experiments showed that carbowax-embedded material gave identical results with those obtained with frozen sections.

The following method of carbowax embedding and cutting was found empirically to be suitable. Pieces of fixed tissue, 2–4 mm. in thickness, were washed thoroughly in running water and embedded, after drying with filter paper, in a mixture of 4 parts carbowax 1540 and 1 part of carbowax 4000 for 3–5 hours in a paraffin oven at 58°. Sections 8 μ thick were picked up from water on albuminized slides and affixed to them directly or by Ivanoff's method.

In some experiments paraffin-embedded sections were used. The various methods of fixation and the treatments given to the sections are described with the experiments.

EXPERIMENTS DEALING WITH THE PRIMARY IMPREGNATION (OR STAINING) OF AXONS AND MYELIN

The following experiments were performed in order to find out which reduction indicators are suitable for the visualization of axons and myelin and

the effect of some contributing factors on the visualization. The sections were not subjected to the action of secondary reducers.

(a) Treatment of sections by 2% AgNO_3 with subsequent 'fixation' in 3% sodium thiosulphate resulted in a weak impregnation of myelin and a much lighter, hardly visible one, of axons. It may be remarked that Bielschowsky (1904) called this part of his procedure 'Myelinfärbung'.

(b) Treatment of sections by Bielschowsky's silver-diammine solution ('ammoniacal silver nitrate') resulted in impregnation of myelin and a lighter impregnation of axons.

(c) The effect of pH on the staining by silver-diammine solutions was studied by the use of solutions of silver acidified (by HNO_3) or alkalinized (by ammonia). A double strength silver-diammine solution was prepared and aliquots of it were brought to the desired pH by the use of varying amounts of HNO_3 and NH_4OH ; the solutions were then diluted to an equal final concentration of silver. After primary impregnation the sections were fixed in thiosulphate. In agreement with the results of Silver (1942) and Samuel (1953a), neither myelin nor axons were impregnated below pH 5. Myelin was well impregnated in pH ranges above 5. The impregnation of axons began at about pH 6-7, increased with the pH, and was optimal around pH 11-12 (at the upper limit of which lies the pH of Bielschowsky's solution). In the optimal zone the axons were impregnated as intensely as the myelin. Further alkalization of the solution resulted in a rapid decrease of the impregnation of axons and a somewhat slower decrease in the impregnation of myelin.

(d) Impregnation was also attempted by silver methenamine solutions prepared according to Gomori (1953) and brought to pH levels between 7 and 8.5. At the lower pH levels only myelin was stained; at pH 8.5 the axons stained lightly.

(e) Treatment of sections: (1) by 1% osmium tetroxide for $\frac{1}{2}$ -1 hour, (2) by 0.2-1% gold chloride for 3-24 hours, (3) by neutral or alkaline $\frac{1}{2}$ % potassium permanganate solutions for between 10 minutes and 1 hour, or (4) for 2 hours in leucofuchsin (Schiff's solution prepared according to Lillie's prescription, 1954), resulted in staining of nerve cells and myelin, but not of axons.

(f) Treatment of sections by a 1% blue tetrazolium (BT) for 2 hours, and by Tetrazol-Purpur (Bayer) at various pH ranges, resulted in no staining in the neutral range and in a progressively increasing staining of myelin in the pH ranges 8-10. At pH 12 the myelin was stained quite intensely, while the axons were stained much more weakly. (These reagents were kindly supplied by Dr. A. M. Seligman and Messrs. Bayer respectively.)

(g) No visible impregnation occurred by treatment for 24 hours with potassium tellurite, HgCl_2 , $\text{Bi}(\text{NO}_3)_3$, CuSO_4 , CuCl_2 , Pb acetate, and FeCl_3 .

(h) The ferricyanide ferric-chloride reaction gave good staining of axons with a very light staining of myelin. It is interesting to note that this was one of the reactions recommended by Unna (1927) for the visualization of reduction-sites in the body. The technique used was that recommended by

Chèvremont (as described by Pearse, 1953) for the demonstration of SH groups.

Conclusions

The results obtained with the reduction indicators tested were of three types:

(a) No staining was obtained with: silver-diammine solutions below the pH of 5, potassium tellurite, HgCl_2 , $\text{Bi}(\text{NO}_3)_3$, CuCl_2 , Pb acetate, and FeCl_3 .

(b) Staining of myelin only was obtained with: silver-diammine between pH 5-6 to 7, silver methenamine below pH 8, osmium tetroxide, gold chloride, KMnO_4 , leucofuchsin and tetrazolium salts below pH 11.

(c) Staining of myelin and of axons was obtained with: AgNO_3 (axons very light), Ag diammine at pH levels above 6-7, tetrazolium salts at pH levels above 11, and Chèvremont's reagent.

These results indicate that myelin can be oxidized more easily than axons under ordinary conditions.

It must be noted here that the lack of staining by some of the indicators does not necessarily mean that the redox potential of the indicators was not high enough for the structure concerned. In some instances the reduced forms of the compounds might have been soluble, in other cases proof will be given below to show that the reduced forms of the compounds, although precipitated, were invisible microscopically.

EXPERIMENTS DEALING WITH THE EFFECT OF REDUCTION AND TONING ON THE METALLIC IMPREGNATIONS

In order to study the effect of reducing agents and of gold toning on the primarily impregnated sections, the following experiments were carried out:

(a) Sections treated by AgNO_3 at different pH levels, or by Ag-urotropin, or by Ag-diammine, or by AgNO_3 for 24 hours followed by Ag-diammine (the procedure used in Bielschowsky's method), were put into a reducing bath of either formalin, pyrogallol, or hydroquinone. The sections were removed from the reducing baths at different times and fixed in thiosulphate.

Whenever the reduction was not unduly prolonged, an existing (even slight) impregnation of axons was markedly increased. The intensity of the impregnation of myelin remained practically unchanged. A prolonged reduction resulted in a complete blackening of both axons and myelin in these cases. No length of reduction could render visible completely unimpregnated structures.

(b) Sections impregnated by various silver solutions were treated with 0.2-1% gold chloride for 4-16 hours; this had the following dual effect. Those structures which were strongly impregnated already changed their colour and appeared more strongly stained; on the other hand, the background staining, i.e. the colour of the less strongly impregnated areas, became much paler.

(c) Sections which were treated for 24 hours in HgCl_2 , $\text{Bi}(\text{NO}_3)_3$, CuSO_4 , CuCl_2 , Pb acetate, and FeCl_3 , were exposed to gold chloride as above. This treatment resulted in an intense impregnation of axons and a slight one of myelin in the case of HgCl_2 -treated sections. In those treated with bismuth nitrate the gold chloride visualized myelin and nerve cells. No staining appeared in the other sections.

Conclusions

Reduction strengthened the impregnation of axons much more than that of myelin. Gold toning revealed that HgCl_2 and $\text{Bi}(\text{NO}_3)_3$ did impregnate nerve tissue elements, but the precipitates were invisible before the treatment by gold.

EXPERIMENTS DEALING WITH THE EFFECT OF FIXATION ON THE IMPREGNATION

The effect of fixation on impregnation was studied by using many of the methods mentioned above. For the sake of simplicity only the results pertaining to impregnation by the method of Bielschowsky, in which gold toning was omitted, will be reported here, as the results with the other methods were of a corroborative nature.

Good staining of axons, neurofibrils, and nerve cells could be obtained in material fixed for many months in 10% formalin solution neutralized by Sørensen's phosphate buffer or in commercial (USP) formalin solutions, the pH of which ranged between 4.5 and 5.5.

Fixation in 10% formalin solutions buffered to pH values between 2 and 7 resulted in a progressive decrease of the impregnation with duration of fixation in the solutions of lowest pH. Although fixation in commercial unbuffered formalin is known to be only seldom detrimental to the impregnation of axons, pH values below 4 appear to weaken the impregnability. Thus, at pH 3 and below there was a marked decrease in the staining of axons already after three weeks. Fixation in formalin-ammonium bromide (FAB) had the same effect on the staining of axons as fixation in formalin acidified by HCl to the same pH.

A similar effect, i.e. weakening of the impregnation of axons and nerve cells, was also obtained in material fixed in neutralized formalin solutions by treatment of the sections with N/1 HCl at 58° for 15 minutes.

Treatment of tissue sections by N/5-N/100 NaOH for 20 hours at room temperature resulted in a weaker impregnation of the axons the higher the pH.

The impregnation of myelin depended much less on the pH of the fixing solution.

Conclusions

Fixation in strongly acid solutions and treatment by strong acids and bases is detrimental to the visualization of axons.

DISCUSSION

It is clear from the experiments reported above as well as from the data available in the literature (cf. Liesegang, 1911; Holmes, 1943; Samuel, 1953*b*) that the most important step in the impregnation procedures is the primary nucleation.

This process is similar in principle to the formation of the latent image in photography, an image which is considered to consist of nuclei of metallic silver (cf. James and Higgins, 1948). During this step the metal is reduced at certain sites, which are most probably those sites where the reducing groups are concentrated in the tissue. Reduction in one site with subsequent migration and deposition at other sites by adsorptive forces (Zon, 1936) cannot be considered probable. No such phenomenon is known to occur in photographic emulsions, and besides, it seems highly improbable that reduced silver particles which have lost their charge would travel a distance great enough to be resolved by the light microscope.

The possibility (Liesegang, 1911; Seki, 1940; Voigt, 1952*b*) that the impregnation depends on the activity of the tissue as a protecting colloid does not seem probable. Voigt compares the protecting activity of the tissue to the activity of the photographic gelatin layer, but migration of silver in the gelatin does not take place to a sufficient extent to diminish the sharpness of a photographic negative. And the proteins of fixed tissue are denatured and cannot be rightly considered to be in a colloidal solution.

It seems obvious, therefore, that impregnation of any structure depends on the adequate choice of an agent of which the oxidizing potential is sufficiently high for the oxidizable groups present in the structure. The fact that most reagents which stained axons stained also myelin, while of those which stained myelin only a few visualized axons indicates that axons are harder to oxidize than myelin. The behaviour of the tetrazolium salts supports this assumption, as their oxidizing potential is known to depend on their pH. Indeed, in the neutral and near-neutral zone, neither axons nor myelin were stained. At higher pH values the tetrazolium salts stained only myelin. The axons were stained slightly at pH 12.

The fact that potassium permanganate, a strong oxidizing agent, did not stain the axons seems puzzling at first. It should be remembered, however, that the reduction of KMnO_4 to MnO_2 is a step-by-step process, and that microscopical observation reveals only the result of the step in which a soluble intermediate is transformed into an insoluble product (MnO_2). Obviously the oxidizing potential of this step is lower than that of silver-diammine at pH 11–12.

On the assumption that axons are harder to oxidize than myelin, the results obtained with Chèvremont's method and with HgCl_2 followed by gold toning, where axons were stained more intensely than myelin, can best be explained by assuming that the axons, although hardest to oxidize, contain more numerous reducing groups than myelin.

The effect of reduction by formalin, pyrogallol, and hydroquinone in increasing the impregnation of axons without affecting the myelin can be explained on the basis of the current opinions on the nature of photographic development. It is assumed (James and Higgins, 1948) that the primary nuclei of the exposed photographic films may act as tiny electrodes which accept electrons from the reducer and surrender them to the silver ions of the solution, thus reducing the ions to metallic silver. Under the conditions prevailing in the photographic plate the amount of secondary reduction is directly proportional to the amount of primary change (i.e. to the amount of light absorbed), as the film represents a homogeneous medium. In the impregnation procedures, the electrode effect responsible for the secondary impregnation cannot be as effective in the lipid medium of myelin (which has probably a low dielectric constant) as in the axons. Reduction, which is not unduly prolonged, results therefore in a stronger final impregnation of axons than of myelin. The fact mentioned by Voigt (1952*a*) that primary nuclei differ in their composition and location in different primary impregnations, corroborates this conclusion, as any metal particle or reducing group may serve as a nucleus, provided its potential is suitable.

The difference in the dielectric constants also explains Liesegang's finding (1911) that the bleaching of sections by ferric chloride (which transforms black silver into white silver chloride) affects the grey matter before it affects the myelin sheaths. Thus the impregnation of axons may be increased or decreased by the various treatments, the axons reacting much more quickly than the myelin.

The intensification of impregnation by reduction depends obviously on the presence of non-reduced silver ions in the sections. These ions must be bound to the tissue as they are not washed away by rinsing with water. The situation is again similar to that prevailing in the photographic plate where unreduced silver is present, bound to gelatin, and serves as a reserve of silver ions which can be reduced during development. In the tissue sections the silver is probably bound to the tissue proteins forming colourless ionic silver proteinates.

As to the nature of gold toning, it is clear that the gold chloride alone, because of its low oxidizing capacity, can impregnate only the myelin sheaths. Gold toning is therefore not an independent reduction of auric ions by the tissue, but rather the coating by gold of reduced silver particles, i.e. reduction of gold ions by the reduced silver, as in the process of industrial gold plating (cf. Samuel, 1953*c*). The differentiating effect of the gold chloride solution is most probably due to its acidity, which causes dissolution of the smaller silver precipitates, or their transformation into white silver chloride.

Treatment by gold chloride of sections in which both axons and myelin were impregnated, also increased the impregnation of axons more than that of myelin. This effect may also be explained in terms of the lower dielectric constant of myelin.

In the impregnation experiments with HgCl_2 and $\text{Bi}(\text{NO}_3)_3$, it appears

obvious that there was a primary impregnation of tissue structures by invisible precipitates which could be visualized by the subsequent gold toning. The intensive primary impregnation of axons indicates that mercuric chloride has a higher oxidizing potential than bismuth nitrate, and probably also higher than any of the previously studied silver compounds.

As for the effect of acid fixatives on the impregnation of axons, the experimental findings corroborate the findings of Cajal (1910). It has been shown that treatment by acids is detrimental also to sections of fixed material.

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Studies of the Impregnation of Nervous Tissue Elements

II. The Nature of the Compounds Responsible for the Impregnation of Axons; Practical Considerations

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SUMMARY

Axoplasm and axolemma differ in their chemical and physical characteristics. The impregnation of axons is mainly due to a compound which is soluble in hot organic solvents and which appears to be situated in the axoplasm.

Ethylene linkages and hydroxyl groups play no role in the impregnation of axons. Sulphydryl and carbonyl groups appear to be partly responsible. These groups are situated mainly in the axoplasm, although sulphydryls are present also in the axolemma. The impregnation of axolemma depends to a great extent on chemical groups which could not be identified.

The fact that solutions of Ag diammine are more suitable for impregnating axons than those of AgNO_3 might be due to one of the following factors: (1) that the reducing groups of the axons are more easily oxidized at high pH levels; (2) that the complex diammine ion might exert a higher oxidizing activity when reacting with some tissue molecules; (3) that the rate of reaction of the reducing groups of the tissue with the Ag diammine ion might be faster than that with the silver ion.

The Bielschowsky procedure may be carried out successfully on sections of tissue embedded either in paraffin wax or carbowax. With the former procedure care should be taken to minimize contact with mixtures of hot solvents. A further intensification of impregnation of paraffin sections is obtained by treating sections for 24 hours in 4% formaldehyde prior to the Bielschowsky procedure.

INTRODUCTION

IN the previous article (Wolman, 1955) evidence was given that the metallic impregnation of axons depends on the reducing capacity of their constituents. Such a capacity depends on the presence of molecular groups having suitable redox potentials.

The present paper deals mainly with the nature of these groups and with the solubility characteristics of their parent molecules. Some inconsistencies, real and apparent, between current opinions and the data emerging from these experiments are also discussed. Finally, some practical aspects of the study for the staining of axons are indicated.

The techniques used in this study are basically similar to those reported in the previous article.

SOLUBILITY CHARACTERISTICS OF THE COMPOUNDS RESPONSIBLE FOR THE IMPREGNATION

In formalin-fixed, carbowax-embedded sections kept for 24 hours in absolute ethanol, ether, or ethanol-ether mixture (1:1), axons could be stained [Quarterly Journal of Microscopical Science, Vol. 96, part 3, pp. 337-341, 1955.]

by the Bielschowsky procedure as well as by Cajal's gold-sublimate method. In some cases (e.g. after absolute alcohol extraction) the impregnation was stronger and sharper than in the controls, probably because of the defatting of the sections which allowed better penetration (cf. Liesegang, 1911; Foot, 1932).

Extractions at room temperature with pyridine, ethanol-xylol (1:1), methanol-chloroform (2:1), ethanol-chloroform (1:1), or ethanol (24 hours) followed by xylol (24 hours) resulted in a somewhat decreased impregnation. This decrease was mainly at the expense of the interior of the axons (axoplasm), while the axolemma was much less affected. Extraction by hot methanol-chloroform and hot pyridine for 3–4 hours at 56° markedly reduced the intensity of the impregnation of axons, and especially of the axoplasm.

The staining of paraffin-embedded sections by Bielschowsky's procedure is known to give erratic, often highly unsatisfactory results. It has been found that results could be greatly improved if: (a) the blocks were passed directly from the clearing fluid into paraffin without a xylol-paraffin or benzol-paraffin hot bath; (b) the block was sufficiently thin to require only a short immersion in the clearing agent-paraffin mixture at a high temperature; and (c) the block of tissue was dried on filter paper before being put into the paraffin oven.

Conclusion

The impregnation of axons is due in part to a lipid constituent which is soluble in hot solvents and is located mainly in the axoplasm.

ATTEMPTS TO CHARACTERIZE THE ACTIVE GROUPS RESPONSIBLE FOR THE IMPREGNATION OF AXONS

The effect of the following reactions on the subsequent impregnation of axons by the Bielschowsky procedure was studied.

(a) *Effect of reagents which react mainly with ethylenic linkages.* Immersion of the sections in 1% OsO₄ for 1–24 hours resulted in a marked staining of the myelin sheaths, but not of the axons. This treatment did not interfere with the subsequent impregnation of axons.

Bromination for 1–4 hours in bromine vapours or in a 10% solution of bromine in CCl₄ (Lillie, 1954) markedly reduced the impregnation of myelin, but did not affect the impregnation of axons to the same extent.

Staining by the performic acid-Schiff reaction (Lillie, 1952) did not render axons visible.

(b) *Effect of reagents which react mainly with hydroxylic groups.* Although acetylation by acetic-anhydride-pyridine mixture for 24 hours at room temperature, and by pure acetic anhydride under the same conditions resulted in a decreased impregnation of axons, the failure of treatment by NaOH, both in aqueous (McManus and Cason, 1950) and in alcoholic solutions, to reverse this effect, proved that the decreased impregnation was due to the dissolving capacity of the reagents. Benzoylation by a 10% solution of benzoyl-chloride in dry pyridine for 24 hours (Pearse, 1954) did not influence the impregnation

of axons either, except for the dissolving effect of pyridine. Similar results were obtained with phenyl-isocyanate. Sections which were dried in the air and then kept for 3 days in a 0.5% solution of the reagent in xylol, followed by thorough washing in xylol, and then by impregnation, did not differ markedly from sections which were kept in xylol only and then impregnated.

Diazotization of phenolic rings by tetrazotized orthodianisidine following the technique used by Seligman (cf. Ashbel and Seligman, 1949) or by diazotized sulphanilic acid (Lison, 1953, p. 417) did not affect the impregnation of axons.

(c) *Effect of reagents which react mainly with sulphhydryl groups.* Treatment by 0.1 M iodoacetic acid for 24 hours at room temperature and by Lugol's solution for 2 hours greatly reduced the intensity of impregnation of the axons. This reduction was at the expense of both axolemma and axoplasm. It has already been noted that treatment by ammonium sulphide increased the intensity of the impregnation of axons.

Attempts at staining of axons by Seligman's DDD reagent resulted in a very faint or no staining of axons. (The reagent was kindly supplied by Dr. A. M. Seligman of the Beth Israel Hospital, Boston, Mass.) Tetrazolium salts at high pH levels stained axons (see Wolman, 1955).

(d) *Effect of reagents which react mainly with carbonylic groups.* Blocking of carbonyl groups by aniline chloride and phenylhydrazine (Lillie, 1952), hydroxylamine and sulphanilic acid (Pearse, 1954) decreased the intensity of impregnation of the axons. The decrease appeared to involve only the axoplasm. Schiff's leucofuchsin did not stain axons.

(e) *Effect of oxidizing agents.* Oxidation by periodic acid (1% for 2 hours), by chromic acid (4% CrO_3 for 1 hour), and by potassium dichromate (3% for 1 and 5 days), followed by thorough rinsing and then by the Bielschowsky procedure, resulted in some increase in the impregnation of axons.

(f) *Effect of reducing agents.* Treatment of the sections by 10% formalin for 24 hours resulted in an intensified impregnation of axons. This intensification was especially apparent in sections embedded in paraffin. A similar but less striking effect was obtained by immersing the slides for 5–10 minutes in a 5% solution of $(\text{NH}_4)_2\text{S}$.

DISCUSSION

(a) *Characteristics of the compound responsible for the impregnation.* The experiments reported indicate that the reducing groups responsible for the reduction of silver ions are not ethylenic links or hydroxyls. Two types of groupings, viz. carbonyls and sulphhydryls seem to be important for the impregnation of axons. Romanes (1950), who conducted a limited number of tests along the same lines, could not find evidence that sulphhydryl groups were responsible for the impregnation of axons, and believed that aldehydic groups were of major importance.

The results of the extractions by lipid solvents and of the blocking of some reactive groups yielded further information. The axolemma and the axoplasm

appear to differ in their chemical constitution. The axoplasm contains a compound which can be dissolved by hot lipid solvents. Its staining in carbowax embedded and in frozen sections appears to be due largely to its content of carbonyl and sulphydryl groups. The fact that techniques which are considered specific for these groups do not visualize axons satisfactorily might be due to their low sensitivity.

The impregnation of the axolemma, on the other hand, appears to depend only in part on the presence of sulphydryl groups. Other chemical groups which cause the reduction of silver ion in the axolemma remain unknown.

Periodic acid oxidation (Lhotka, Myhre, and Combs, 1953; Lhotka and Myhre, 1953) and chromate oxidation (Golgi's method, cf. Kallins, 1926) are known to increase the impregnability of tissue constituents. The means by which these oxidative agents increase the intensity of axon impregnations can be deduced from known facts. Chromate and periodate oxidation may result in the formation of new carbonyls, and this effect may compensate for a possible loss of other reducing groups which have been oxidized.

The effect of the reducing agents tested might be due to reduction of labile oxidation-reduction systems (such as sulphydryl or polyphenol groups), or in the case of formaldehyde to the formation of reducing groups by the effect of formaldehyde on some lipids (cf. Wolman and Greco, 1952).

(b) *The oxidizing activity of silver diammine solutions.* The factors regulating the oxidizing potential of silver diammine solutions have been discussed by Nauta and Gygas (1951) and by Garven and Gairns (1952). These authors suggested that the oxidizing potential of the solutions is due to dissociation of the complex Ag-diammine ion into Ag^+ and ammonium ions. The oxidizing potential was supposed to depend on the minimal concentrations of silver ions present in the solution. In support of their thesis Nauta and Gygas reported that silver diammine solutions which contain more Na^+ ions (up to a given optimum) impregnate sections more strongly than pure Ag-diammine solutions (prepared by adding NH_4OH to AgNO_3 solutions). These data could not be substantiated, and, furthermore, the theory does not account for the fact that Ag-diammine solutions, in which the concentration of Ag^+ ions is considerably lower, impregnate structures more strongly than AgNO_3 solutions.

In the preceding paper it was implied that Ag-diammine solutions act as stronger oxidants than AgNO_3 solutions, but this assumption appears contrary to the known data of physical chemistry, which assign a higher oxidizing potential to AgNO_3 .

These difficulties cannot be explained in a final manner, although three possibilities may be suggested.

(a) It is possible that axons are higher on the oxidation-reduction scale, that is: can be oxidized by stronger oxidants only than myelin in the neutral and nearly neutral pH range, while in the alkaline range they are more easily oxidized than myelin. Such behaviour is known in hexoses which are much more easily oxidized in an alkaline pH.

(b) The complex diammine ion may behave abnormally when reacting with complex tissue molecules because of participation of electrons not belonging to the external orbit. (A suggestion made to the author by Dr. A. Katchalsky of the Department of Physical Chemistry.)

(c) Ag-diammine, although of a lower oxidation potential than AgNO_3 , might be more reactive than AgNO_3 towards the reducing groups of the axons. In other words, while both solutions have a high enough potential to oxidize axons, the reaction-rate with Ag-diammine would be faster.

PRACTICAL APPLICATIONS OF THE STUDY

Axons can be impregnated by the standard Bielschowsky procedure in carbowax embedded material after fixation in formalin. The advantages of carbowax embedding over frozen sections include the ease of handling and cutting the sections and the uniformity of results, as the whole section is immersed and air bubbles are eliminated.

The results may be somewhat improved by extracting the sections with alcohol for 24 hours, or with 20% pyridine in alcohol for 10–30 minutes.

Axons can be demonstrated in paraffin embedded formalin-fixed material, provided that the tissue is not immersed in a hot clearing agent (xylol, benzol, &c.) and the period of immersion in the mixture of alcohol-clearing agent be kept short. The period in which paraffin replaces the clearing agent should be as short as possible.

The impregnation is markedly improved if sections of paraffin-embedded tissue are left for 24 hours in 10% formalin, then rinsed before the standard Bielschowsky procedure is carried out.

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Dermal Nerve-endings in *Rana* and *Bufo*

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SUMMARY

In the skin of *Rana* and of *Bufo* there are certain coarse medullated nerve-fibres which give off unmyelinated branches dividing repeatedly to form richly varicose endings at various levels, but mainly in the *stratum spongiosum* of the dermis. There is no capsule. These fibres are of a type distinct from those medullated fibres which end freely in the epidermis. Their function is discussed: it is possible that they are fast nociceptive fibres.

IN the skin of the frog, *Rana temporaria*, and of the toad, *Bufo bufo*, there are certain coarse medullated nerve-fibres which, instead of passing to the epidermis, give off unmyelinated branches which divide repeatedly to form



FIG. 1. Frog, thigh, whole mount: myelinated fibre with varicose endings in the *stratum spongiosum* of the dermis. Camera lucida drawing. Methylene blue. $\frac{1}{12}$ -inch oil immersion objective, $\times 6$ eyepiece.

richly varicose endings in the dermis. Two such endings of a fibre in the thigh of a frog are illustrated in fig. 1. There is no capsule, nor is the course of the varicose branches similar in any two endings; the branching may be richer and the spread of the unmyelinated portion more extensive than in those shown. These fibres have been reported before by Hulanicka (1912), who described fibres running in the deep layers of the dermis of the thigh of *Rana temporaria* which gave off '... des filaments couverts de varicosités arrondies' [Quarterly Journal of Microscopical Science, Vol. 96, part 3, pp. 343-349, 1955.]



FIG. 2. Frog, knee: diagrammatic section based on camera lucida drawings showing the course of a single myelinated fibre and the depth in the skin of selected varicose endings. Methylene blue. $\frac{1}{8}$ -inch objective, $\times 6$ eyepiece.



FIG. 3. Toad, pectoral region, whole mount: myelinated fibre with varicose branches lying beneath a granular gland. Camera lucida drawing. Methylene blue. $\frac{1}{8}$ -inch objective, $\times 6$ eyepiece.

qui se divisent et se subdivisent en formant des grappes d'une grande élégance'. It scarcely needs the accompanying drawing to make sure that these fibres are of the same type; Hulanicka also described a dermal ending in the connective tissue of the thumb-pad of the male which she said was partly

encapsulated, although the illustration looks very like the other, varicose, endings.

In the present instance most of the material was prepared, like Hulanicka's, by the subcutaneous injection of methylene blue. The skin was removed and placed in ammonium molybdate an hour after injection with a 0.01% solution of methylene blue in Ringer's fluid or in isotonic saline. Some of the toads received a previous injection of hyaluronidase, to aid penetration of the stain (see Pallie, Corner, and Weddell, 1954). Frog skin was also fixed in 8%

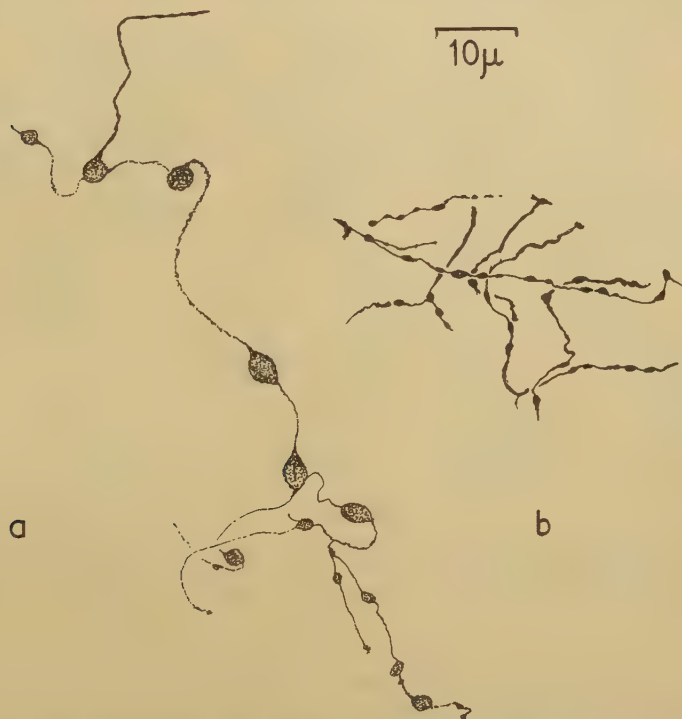


FIG. 4. Frog, thigh, whole mount: (a) portion of a varicose ending in the *stratum spongiosum*; (b) part of the motor plexus on the proximal side of a granular gland. Camera lucida drawings, Glees's modification of Bielschowsky. $\frac{1}{12}$ -inch oil immersion objective, $\times 6$ eyepiece.

formaldehyde for impregnation by Glees's modification of the Bielschowsky method (Glees, 1946), and in Heidenhain's 'Susa' for sectioning, and impregnation by Holmes's silver nitrate method (Holmes, 1943).

The depth in the skin of the varicose endings varies from the upper levels of the *stratum compactum* to (in exceptional cases) the lower levels of the epidermis; most are in the *stratum spongiosum*. Fig. 2 represents the positions of selected endings of a single fibre as seen in sections of the skin; the diagram was constructed from sections of an area which had been previously mapped in a whole mount. It was unusual for such a fibre to enter the epidermis, though many endings lay just beneath it in the upper layers of the *stratum spongiosum*.

The unmyelinated portions of the fibres in question often lie close against the walls of the skin glands, particularly in those areas where the *stratum spongiosum* is closely packed with glands. In *Bufo* the best-stained varicose fibres were usually seen on the under surface of the large granular glands (fig. 3); but many of the endings are remote from any gland. The motor-fibres to the glands (described for instance by Coghill, 1899) are especially well seen in *Bufo*, though their arrangement is similar in *Rana*, and are readily distinguished from the varicose endings of the myelinated fibres. On

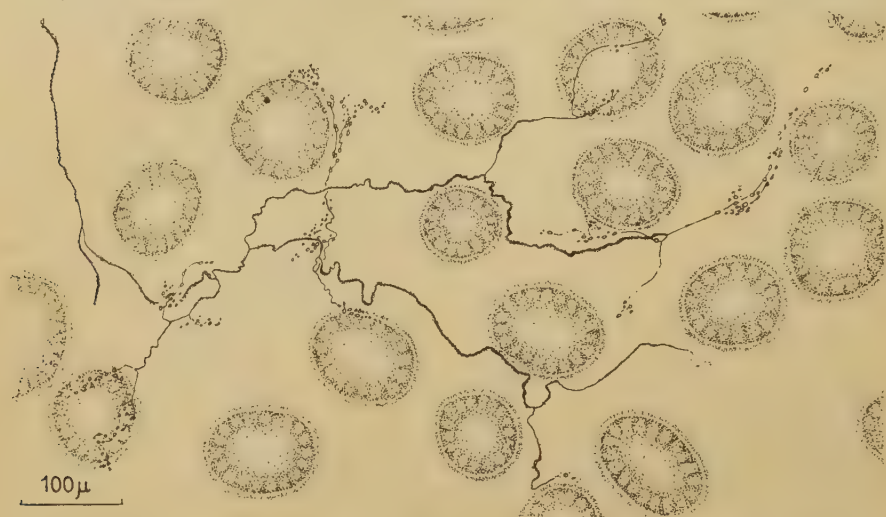


FIG. 5. Frog, thigh, whole mount: optical section of the *stratum spongiosum* showing part of the course of a single myelinated fibre with varicose endings, and the positions of the mucous glands. Camera lucida drawing. Methylene blue. $\frac{1}{4}$ -inch objective, $\times 6$ eyepiece.

the wall of a granular gland is a plexus of fine unmyelinated nerve-fibres which come to run on the long axis of each smooth muscle-cell, so that though on the proximal side of the gland their arrangement is somewhat irregular, they run distally towards the neck of the gland as neatly as the longitudes on a globe. The varicose endings, though they may be applied closely to the wall, have no such regular arrangement: fig. 4 shows a varicose ending (*a*) from a Bielschowsky preparation of frog skin compared with a portion of the innervation of a granular gland in the same preparation (*b*). The mucous glands lack a smooth muscle coat, and the fine fibres on their walls form an irregular plexus; in fig. 5, from the thigh of *Rana*, the positions of some of the varicose endings of a medullated fibre, and of the mucous glands, are indicated.

The varicosity of the endings is so striking that it deserves consideration. In methylene blue preparations, stained for an hour *intra-vitam* and subjected to considerable handling before fixation, the irregularity of the unmyelinated axon cylinder may well be artificial; the question is discussed by Weddell and Zander (1950 and 1951). Unmedullated fibres are frequently

varicose: fig. 6, left side, shows a portion of a sub-dermal nerve-bundle of *Rana* containing unmyelinated fibres, some of which are varicose, and also coarse medullated fibres, one sending a branch through the *stratum compactum* and giving off varicose branches in the *stratum spongiosum*, as shown in the right-hand side of the drawing. The free endings of fibres running in the epidermis may also be varicose, though generally less so. It is noteworthy, however, that the dermal varicose endings have a precisely similar appearance in Bielschowsky preparations of formalin-fixed material (fig. 4) and also in



FIG. 6. Frog, thigh, whole mount: left side of line, sub-dermal level, right side of line, *stratum spongiosum*; nerve-bundle of the sub-dermal plexus with a myelinated fibre branching and forming varicose endings. Camera lucida drawing. Methylene blue. $\frac{1}{2}$ -inch oil immersion objective, $\times 6$ eyepiece.

material fixed in Heidenhain's 'Susa' and impregnated by Holmes's method. Possibly a large unmyelinated axon is particularly unstable, though long stretches of the unmyelinated portions of the fibres may be smooth and unswollen, even when richly varicose elsewhere. It is not suggested that the varicosities cannot be artifacts, nor that they have any functional significance, but they are so constant in occurrence that they provide a useful means of recognizing the fibre type and justify the description of the endings as varicose.

It should be considered whether the nerve-endings described do in fact constitute a distinct type of fibre, or whether they might not be, for instance, partially stained epidermal fibres, or the growing ends of fibres which will eventually enter the epidermis. Another possibility is that they are epidermal

fibres undergoing retrogression, though this is made improbable by the richness of the branching of the unmyelinated portions of the dermal fibres; all the skin fibres branch repeatedly, but in the case of the large medullated fibres, dividing only at the nodes, the branches are given off at longer and more regular intervals than in the unmyelinated varicose endings. The varicose endings are numerically abundant and of constant occurrence, having been seen in frogs killed throughout the summer months from April to September, and also in February and December, so that it is also unlikely that they are growing fibres; in any case the seasonal changes connected with breeding affect mainly the tactile papillae of the upper surface (Hulanicka, 1912), and there is no reason to suppose the varicose fibres are concerned in this activity. As to partial staining, it can never be certain that any fibre is stained right to its end, but the change in character of the fibre as it loses its myelin sheath, often deep in the dermis, makes it irrelevant whether the unmyelinated portion does in fact continue beyond the part stained or not. It is accordingly considered that the dermal varicose endings are of a type distinct from the epidermal free endings.

Most of the endings illustrated from *Rana* are from the skin of the under-side of the thigh, which is of convenient thickness and relatively little pigmented; similar endings were found on all parts of the legs and on the forelimbs and on the back and sides. In the mid-ventral region the presence of dense layers of iridocytes made it difficult to trace fibres and no varicose endings were seen, though they were found more laterally on the belly and throat. In *Bufo* they certainly occur ventrally (fig. 3 is from the pectoral region). Their distribution is therefore probably general.

The calibre of the fibres where myelinated was of the same order as that of the coarse medullated fibres with free endings in the epidermis: in preparations in which both types of fibre were stained they could be distinguished only by tracing the branches to their endings, as at comparable levels in the skin the diameters appeared the same. With this material, no accurate determination of fibre diameter could be made, but at the sub-dermal level it was about $5\ \mu$ (see fig. 6); as the fibres branch the diameter decreases, and might therefore be greater in the nerve-trunks approaching the skin.

There has been some confusion in the literature over the question of specifically dermal nerve-endings (other than fine unmyelinated fibres) in anuran skin: Merkel (1880) claimed that myelinated nerve-fibres ended in close association with certain cells ('Tastzellen') in the dermal papillae of the tactile spots of frogs and toads. Other authors, notably Eberth and Bunge (1892) and Hulanicka (1909) on *Rana*, and Fahrenholz (1929), working on the well-developed tactile papillae of *Xenopus*, state that the fibres in fact pass through the dermal papillae to end in the epidermis, as one would expect from the physiological evidence. Adrian, Cattell, and Hoagland (1931) state that the receptors for light touch in frog skin are large medullated fibres ending in the epidermis, for when the epidermis was scraped away the usual tactile discharge was no longer obtainable. The application of weak acid, or of

various noxious stimuli, gave rise to slow impulses, which were the only type of impulse obtained after removal of the epidermis; these would be mediated by fine fibres. Rubin and Syrocki (1936), using Cajal's silver nitrate method, claimed that simple bulb-like endings were present in the dermis of *Rana pipiens*; no such nerve-endings have been seen in the present study.

Though there are reports of the existence of a fast-fibre system responsive to noxious stimuli in mammals (Adrian, 1931; Zotterman, 1933) the older literature is vague about the existence in frog skin of fibres of large calibre, other than the tactile receptors of the epidermis. More recently Maruhashi, Mizuguchi, and Tasaki (1952), have made a detailed study of the impulses from single afferent fibres from toad skin, though without any reference to the position of the receptors in the skin. They found coarse fibres responsive to stimuli other than light touch, namely, fibres of estimated diameter 6 to 9 μ which were excited by pinpricks; there were also a few fibres of diameter 4 to 5 μ responding to pressure. The diameter of the tactile fibres was from 8 to 15 μ , which overlaps with that of the large nociceptive fibres. Mr. A. S. Jarrett reports (personal communication) that he has on two occasions come across nociceptive fibres in *Rana*, of a diameter comparable with that of the tactile fibres, though somewhat smaller.

On the evidence available one cannot come to any conclusion as to the function of the fibres with varicose endings, not even that they are functionally all of one type, except that, since they are not motor fibres innervating the skin glands, they are presumably sensory. It is tempting to suppose that they may be the large nociceptive fibres mentioned above, but the evidence is not in any way conclusive, particularly as the estimates of diameter by histological and physiological methods were carried out at different points on the nerves, and in different species.

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The Lateralis Organs and their Innervation in *Xenopus laevis*

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With one plate (fig. 4)

SUMMARY

The innervation and general morphology of the lateralis organs of *Xenopus laevis* are described. Each group of organs is innervated by two large and two or fewer small fibres. Each of the large fibres gives off a branch to each of the lateralis organs in the group, but the branching of the small fibres is less regular. Each lateralis organ is therefore innervated by two large and up to two small fibres. The large fibres branch extensively at the base of the organ, the small fibres less so, and the nerve endings lie basal to or between the sensory cells. No terminal swellings could be seen. The endings of a particular fibre are not restricted to any one region of the organ.

No special function could be assigned to the dual arrangement of the innervation.

The original accounts of the structure of the lateralis organs were adequate. The recently suggested division of the supporting cells into three types is not justified.

It is probable that a cupula exists on the lateralis organs but it could not be observed. It is suggested that the supporting cells secrete the cupula.

INTRODUCTION

XENOPUS is one of the few Anura in which a well-developed and functional lateralis system is retained after metamorphosis, and this condition may be correlated with the retention of an aquatic way of life. Maurer (1895) first recognized the sensory function of the lateralis organs and his description of their structure and arrangement has scarcely needed amplification since. Grobelaar (1924) and Escher (1925) added little new in their accounts, except that the latter brought the lateralis system of *Xenopus* into relationship with those of other Amphibia. The changes in the distribution of the lateralis organs over the body at metamorphosis were followed by Paterson (1939) and a previously unnoticed median ventral row of organs was described by Elkan and Murray (1951).

Kramer (1933) has shown that the function of the lateralis organs is the detection and precise localization of minute water disturbances such as would be made by the wriggling of small food animals. The great sensitivity of the lateralis system has been confirmed by Dijkgraaf (1947) who was, however, especially interested in the physical nature of the stimulus. Both these workers employed a behavioural method.

The innervation of the system of lateralis organs of each side is by two nerves of cranial origin (van der Horst, 1934; Paterson, 1939); the first, or anterior lateralis nerve, runs from a nucleus in the wall of the fourth ventricle

separate from the roots of V, VII, and VIII (although its ganglion fuses with that of V and VII); most of the fibres separate out again distally and continue as the tractus supraorbitalis to the organs of the head and snout; a few run ventrally in the mixed ramus mentalis VII. The second, or posterior lateralis nerve, runs from a nucleus immediately posterior to that of the anterior nerve, forms a mixed ganglion with the vagus, then separates out again and runs to the skin alongside the main cutaneous blood-vessels. The details of the innervation near and in the lateralis organs have not hitherto been investigated.

A trematode infection of the skin of *Xenopus*, which was restricted to the locality of the lateralis organs, was described by Elkan and Murray (1952).

Those properties of the lateralis organs which have not been directly studied in *Xenopus* may be deduced from the properties of the homologous lateralis organs in other amphibians and fish. The embryology and experimental regeneration of the lateralis system are the main subjects of a review by Wright (1951) and the structure and function of the whole acoustico-lateralis system are surveyed by Dijkgraaf (1952). Details of the innervation of the lateralis organs have been studied in urodeles, but the results are contradictory. Retzius (1892) and Larsell (1929) both described the nerve-fibres as ending extracellularly between the sensory cells at the level of their nuclei; terminal swellings were found. On the other hand, Charipper (1928) and Chezar (1930) held that the endings were intracellular, with the fibres entering the bases of the sensory cells and ending in a cup-shaped network about the base of the nucleus.

My investigation has been carried out in order to provide the anatomical background for an electrophysiological study of the function of the lateralis organs in *Xenopus laevis* (Daudin) (which will be published elsewhere). Certain of the electrical results are of interest in connexion with the innervation and will be referred to in the discussion.

The arrangement and structure of the lateralis organs

The lateralis organs are grouped together in small ridges or plaques which are arranged over the skin in rows, each row corresponding to a lateral 'line' in fish. The distribution of the plaques and the nomenclature of the rows adopted in this account are shown in fig. 1. In each plaque there are from three to twelve lateralis organs, arranged along its length alternately with tactile organs (Calabresi, 1924) (figs. 2 and 4, A). Each lateralis (or neuromast) organ consists of a rounded group of cells embedded in the epidermis, open to the external medium at the surface and separated from the basement membrane of the epidermis by a tenuous layer of epidermal cells (figs. 3 and 4, B). The opening at the surface is oval (with its long axis transverse to the long axis of the plaque) and is usually sunk slightly, and the base of the organ and therefore the basement membrane of the epidermis may extend down into the stratum spongiosum of the dermis. The dimensions of a typical organ are $70\ \mu$ from base to apex, $70\ \mu$ in the direction of the length of the plaque, and $100\ \mu$ across the plaque.

Each lateralis organ contains two types of cell, sensory and supporting. The sensory cells, which are secondary sense cells, are pear-shaped and lie in the centre of the organ with the so-called sensory hairs at their apices; the elongated supporting cells occupy the rest of the organ on each side of and basal to the sensory cells, with their nuclei forming a layer round the base of the organ.



FIG. 1. The arrangement of the lateralis plaques in *Xenopus laevis*. Dorsal and ventral views of a typical adult female.

METHODS

Pieces of skin of recently pithed *Xenopus* were prevented from rolling up by laying them on card, and were then fixed. Nerve axons in serial paraffin sections were stained by Holmes's (1947) method after fixation in a 4% solution of formaldehyde in a saturated solution of mercuric chloride. Since this method did not prove very satisfactory in demonstrating the extreme tips of the fibres even when the buffer solution was of pH 8.7 or 9.0 (suggested by Holmes—personal communication), a modification of Bielchowski's method and a methylene blue method were attempted, but the initial results were not promising and the methods were not followed up. Other special techniques employed will be mentioned in their context.

OBSERVATIONS

The innervation of the lateralis plaques

Maurer (1895) stated that each plaque was supplied by its own small branch of the local lateralis nerve. This innervation has now been studied in greater detail. A typical plaque is innervated by two myelinated fibres of diameter 8–15 μ . Each of these fibres divides, and gives a branch to each of the lateralis organs of the plaque, with the result that each organ is supplied by two fibres. The arrangement of this branching is very regular and may even present a

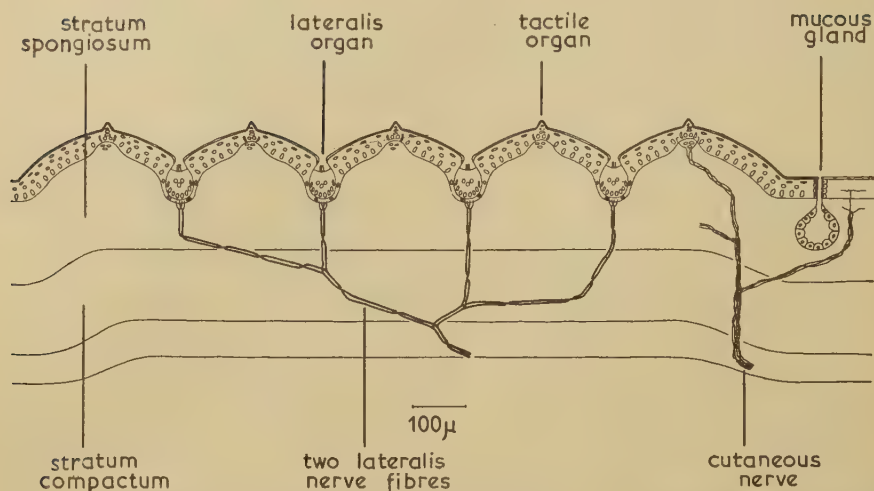


FIG. 2. A lateralis plaque, showing the arrangement of the lateralis and tactile organs and their nerves (diagrammatic longitudinal section).

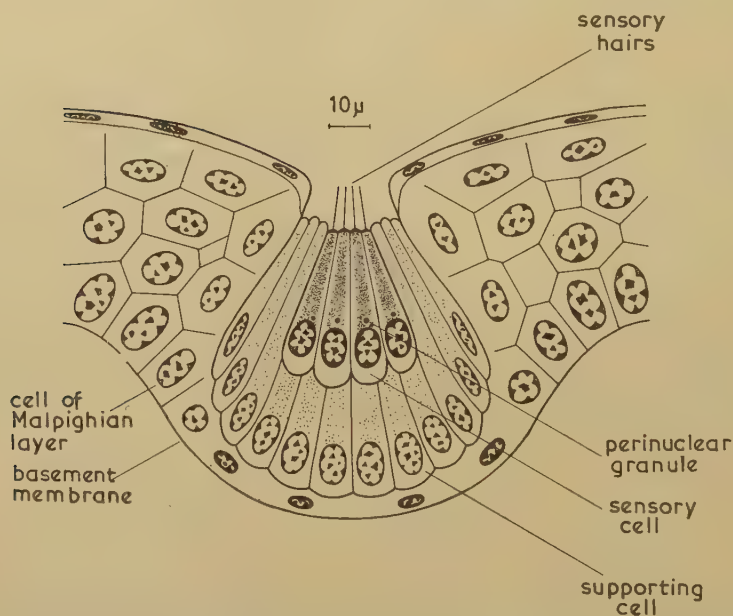


FIG. 3. A single lateralis organ, showing its situation in the epidermis and the relationships of the sensory and supporting cells (diagrammatic).

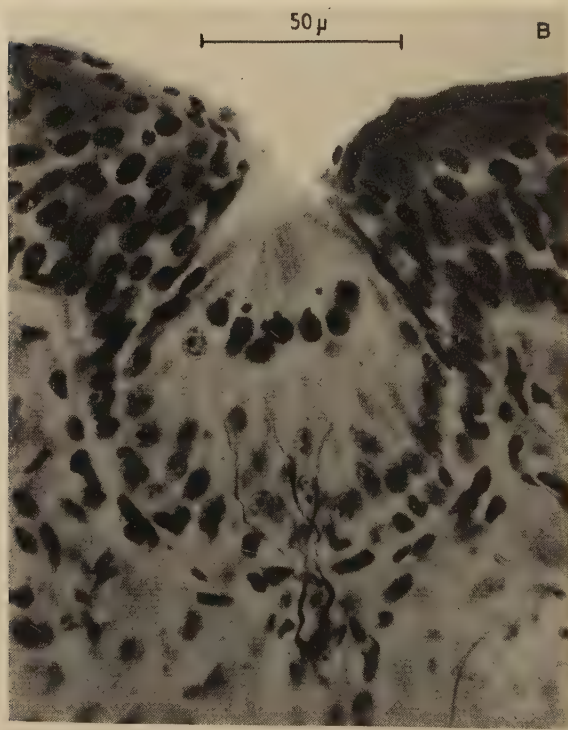
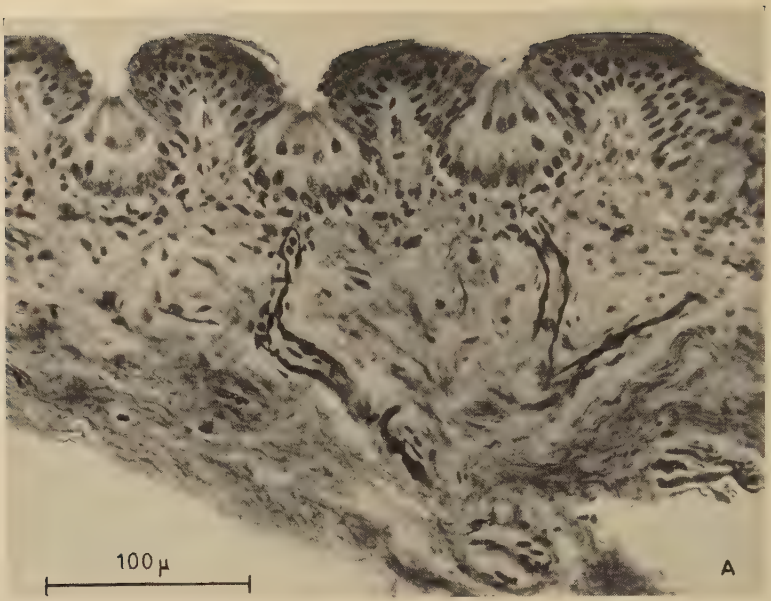


FIG. 4
R. W. MURRAY

'tramline' appearance (figs. 2 and 4, A). This two-fibre system has been traced in serial sections of individual plaques from the circumorbital and maxillary rows, from the anterior and posterior regions of the middle lateral row, and from the median ventral row.

As well as by the two large fibres, the plaque may be innervated by a variable number of small, myelinated fibres of diameter $1-5\ \mu$. In those plaques which were most intensively studied the number of small fibres was two (four plaques), one (two plaques), or none (three plaques).

As a check on the number of large fibres which reach the plaques counts were made of the number of fibres in the branch of the lateralis nerve which runs in the skin beneath the posterior end of the middle lateral row. At a point seven plaques forward from the end of the row there were eleven large fibres, and seven plaques farther forward again there were twenty-five. Thus between the two points seven plaques were supplied by fourteen fibres, although the posterior seven had only eleven fibres. This low number may possibly be explained by the small and irregular arrangement of the plaques and by overlap from the other side of the body.

The point at which the fibres divide varies with the distance apart of the two organs to which the fibres run, for the wider apart the organs are, the deeper in the dermis does the point of division occur. This fact and the regularity of the 'tramline' arrangement are correlated with the way in which the organs of the plaque arise by budding or fission (Escher, 1925; Stone, 1933; Speidel, 1948) from a single organ in the larva. The original organ must therefore have had a double innervation, and when it divided both the fibres must also have divided; this is in agreement with the fact that there is no localization of the endings of either fibre in any special region of the sense organ (shown most clearly in fig. 5). The two large fibres which finally run to any particular organ may divide up still further almost immediately after the fibres to the next organ have branched off (fig. 6), or there may be no further branching until the fibres enter the organ.

The small fibres, on the other hand, are much less regular in their branching, for this may take place more centrally than the point at which the bundles of fibres to the different organs separate. Thus in one instance where there were two large and two small fibres in the nerve-bundle which approached the plaque, the small fibres had divided to give six before the first division of the large fibres had occurred. The total number of endings produced by the large fibres is greater than that produced by the small.

The remarkable, duplicated twisting of the fibres shown in fig. 5 occurs to a lesser extent in other lateralis organs but is not typical of all.

The myelination of the fibres stops just proximally to the level of the base of the organ.

FIG. 4 (plate). A, a lateralis plaque in longitudinal section, showing nerve-fibres in the dermis (Holmes's method).

B, a single lateralis organ, showing part of the innervation (Holmes's method).

The nerve endings in the lateralis organs

The fibres can be traced up to the level of the nuclei in the bases of the sensory cells (figs. 5 and 6); the endings are probably extracellular, but because of the difficulty of impregnating the fine terminal regions it has not been possible to obtain definite proof. Some fibres divide and run in a Y shape round the base of a cell; some run up to the base of a sensory cell, bend to one side, and end between the cells; others may divide more basally and give a branch that runs laterally across the organ, looping under the bases of the sensory cells and ending at the side of the last. Endings that are at first sight

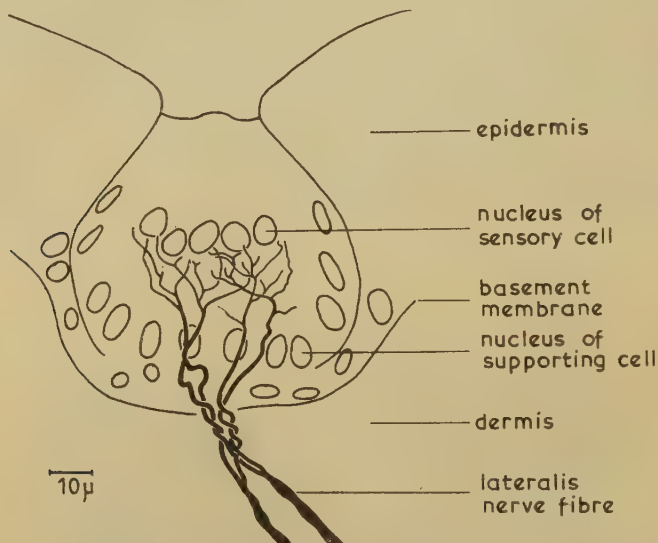


FIG. 5. The innervation of a lateralis organ (reconstructed from serial sections).

intracellular can always be referred by careful focusing to the plane between the sensory cell nuclei.

No terminal knobs could be seen. Dark knob-like enlargements seen through the microscope were either staining artifacts where a fibre had been cut as it left the section or optical artifacts caused by a faint fibre taking a sudden turn perpendicular to the plane of the section.

Cytology

Perinuclear granules (Charipper, 1928) were found in the sensory cells in the region of the nucleus. They were basiphil, did not colour in a chromic acid/Schiff test for polysaccharides, and did not reduce ammoniacal silver nitrate in an argentaffine test (Burtner and Lillie, 1949). In some routine preparations they were surrounded by a narrow, clear region of cytoplasm. In general their staining reactions were similar to those of the apex of the cell at the base of the sensory hair.

The localization and extent of the Golgi element were studied with Kolatchev's osmium impregnation technique (Baker, 1945). Heavy impregnations were easily obtained in the lateralis organs and the main regions concerned lay at each end of the nuclei of the sensory cells and distal to the nuclei of the

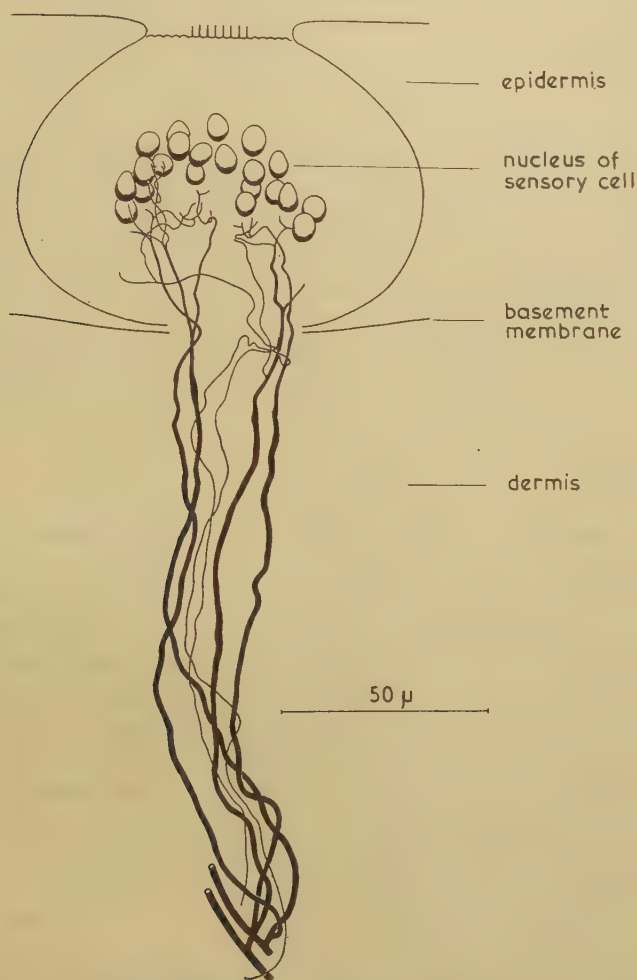


FIG. 6. The innervation of a lateralis organ; the fibres are traced back to their junction with the fibres from the next organ in the plaque (reconstructed from serial sections).

supporting cells. An elaborate network (as Charipper found in *Necturus*) was not sought for or obtained. The relationship between the impregnated regions and the distribution of lipoid material was studied with a Sudan black technique (Baker, 1949). Numerous small (less than $1\ \mu$) droplets were found in both sensory and supporting cells; larger ($1-1.5\ \mu$) crescent-shaped sudanophil bodies occurred in the sensory cells in the region distal to the nucleus. It is

possible that these correspond to the clear region around the granules. A diffuse dark colour of the apices of the supporting cells was also found.

The apical region of the supporting cells had other specific staining reactions: after toluidine blue and Nile blue it appeared red-purple in colour in contrast to the blue of the rest of the organ. There was also a weak positive reaction after a chromic acid/Schiff test, but the pink colour was also found in the apical region of the sensory cells.

The cupula

A cupula could not be observed either in histological preparations or in the living toad. Thick frozen sections of fresh skin were cut and observed by phase-contrast microscopy and also with polarized light; the sections were sticky and difficult to handle, but in one of the less damaged ones a transparent mass projecting from the mouth of a lateralis organ may have represented the remains of a cupula.

Sensory hairs were seen in most preparations, arising from the sensory cells only. (Kramer (1933) described them as also overlying the supporting cells.)

DISCUSSION

The innervation

There are two main problems raised by the anatomical details of the innervation. The first concerns the significance of the presence of two types of fibre, the large and the small. An anatomical and functional differentiation between large and small fibres has been described by Katsuki, Yoshino, and Chen (1951) in lateralis organs of *Anguilla*; they suggested that such a double innervation was typical of all higher sense organs. In *Anguilla* the large fibres innervated the centre of the organ with little branching, whereas the small fibres ended peripherally with much branching. The large fibres did not discharge spontaneously, had a relatively high threshold, and allowed discrimination between stimuli at high intensities. The small fibres had a spontaneous discharge with a low threshold for alteration and reached their maximum frequency at relatively low intensities of stimulation. The morphological requirements of this theory are not satisfied in *Xenopus*, and spontaneity of discharge is not limited to the small fibres.

A second theory of the function of the small fibres is derived from work on the mammalian ear where there are also two sizes of fibre; it is suggested (Galambos, Rosenblith, and Rosenzweig, 1950) that the small fibres may be efferent and not sensory at all. There is no evidence on this point for any lateralis organ.

The second problem concerns the significance of the dual innervation by large fibres of each of the organs in the plaque. The regularity of occurrence of this system indicates that there may be a functional distinction between the two fibres, but this could not be detected in the electrical investigation. A functional double innervation is known in the lateralis organs of *Raja* (Sand,

1937), where the two types of fibres responded to movement in opposite directions of the fluid in the canal. Such a system would not be appropriate in *Xenopus*, for the necessary mechanical limitation of the stimulus to two opposite directions is not present.

Types of supporting cell

In his description of the lateralis organs of *Necturus*, Charipper (1928) suggested a classification of the supporting cells into the three categories of mantle, basal, and sustentacular cells. This subdivision is followed by Chezar (1930), and in her review Wright (1951) implies that it also applies to the lateralis organs of other Amphibia and even fish. The usefulness of such a division of supporting cells depends on the sharpness of the distinctions which can be drawn between the types.

The mantle cells in *Necturus* were found at the edge of the lateralis organs and could be further distinguished from sustentacular cells by two other characters: the cells were elongated with elongated nuclei lying half-way up them, and their apices did not extend so far as those of the other supporting cells so that a marginal furrow was formed separating the organ from the rest of the epidermis. In *Xenopus*, cells with elongated nuclei occur in this position, but they are joined by a series of cells with nuclei of intermediate shape to the more typical supporting cells of the rest of the organ; a marginal furrow is not normally present. A separate category of mantle cells is not therefore justified in *Xenopus*.

The differences between the basal and sustentacular cells in *Necturus* were even slighter, and involved only the position of the nucleus at the base of the cell. In the basal cells the nucleus was terminal, while in the sustentacular cells there was a short 'tail' of cytoplasm and the nucleus was slightly farther from the base. In *Xenopus* a double layering of the nuclei of the supporting cells often does not occur (fig. 5), or, if it does (fig. 4), it has more the appearance given by the nuclei of a pseudo-stratified epithelium, and is the result of the close packing of tall, narrow cells with large nuclei, rather than an indication of two distinct cell types. The original accounts which describe only sensory and supporting cells are therefore entirely adequate for the lateralis organs of *Xenopus*.

The cupula and its secretion

The failure in this investigation to observe a cupula in *Xenopus* must not be taken as proof of its absence. The existence of a cupula (a jelly-like transparent structure in which the sensory hairs are embedded) is characteristic of the majority of the sense organs of the acoustico-lateralis system (Dijkgraaf, 1952) and the occasions on which it has been proved to be absent are few; its transparency and fragility make negative reports hard to substantiate, but Katsuki, Mizuhira, and Yoshino (1951), who have seen a cupula in the lateral line canals of some teleosts, claim that it is lacking in others.

The existence of a cupula in the lateralis organs of Amphibia has never been

definitely established, although in *Rana* the description given by Gaupp (1904) of a hyaline tube in which the sensory hairs lie is suggestive. On the other hand, Speidel (1949) and Wright (1951), both of whom have observed lateralis organs in living *Rana* tadpoles, made no mention of a cupula, and Speidel described separate, stiff hairs arising from the centre of the organ.

The structure of the lateralis organs of *Xenopus* makes possible a localization by histochemical methods of the cells that secrete the cupula (assuming that a cupula is normally present). For in these organs, unlike for instance the ampullae of the semicircular canals, there is a complete restriction of the sensory cells to the centre of the organ, with only supporting cells at the side. The chemical nature of the cupula is not known, but Katsuki, Mizuhira, and Yoshino (1951) claim that it is probably mucoprotein. If this is so the results of the staining with toluidine blue and Nile blue are significant and the restriction of the metachromatic red colour to the supporting cells may be taken as evidence for the secretion of the cupula by these cells. The osmiophil and sudanophil material in the sensory cells indicates secretory activity there as well, but this could be concerned with the elaboration and repair of the sensory hair; the secretory product would collect as perinuclear granules in the region of the Golgi element.

The function of the cupula

In a typical sense organ of the acoustico-lateralis system the cupula plays an important part in the transformation of movements of the fluid medium into an effective stimulation of the sensory cells. Its elasticity and rigidity play a considerable part in determining the range of frequency of vibrations to which the lateralis organs will respond (Katsuki, Mizuhira, and Yoshino, 1951), and affect the damping of the resonant system of the semicircular canals (Groen, Lowenstein, and Vendrik, 1952). If the adequate stimulus is tension applied to the apex of the sensory cell (Jielof, Spoor, and de Vries, 1952), the cupula may be necessary to provide mechanical stiffness which the sensory hairs might not possess by themselves, and would ensure the synchronization of any microphonic generator potentials (Jielof and others) which may arise. The large surface area of the cupula will enable the energy of a large volume of moving water to be transferred to the sensory cells.

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An Easily Made Tissue Culture Perfusion Chamber

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With one plate (fig. 3)

SUMMARY

A perfusion chamber is described with which a tissue culture can be irrigated successively with different immersion media. Only 1-2 ml. of medium are required for each change, which can be accomplished within 1 minute. Simplicity of design permits speed and economy in manufacture. Perfusion by suction instead of by positive pressure has eliminated certain drawbacks of previous designs. Mixing effects in the chamber have been studied. Any component of an immersion medium can be reduced to 0.5% of its initial concentration by the use of only 2 c.c. of fresh medium. The chamber has been used in the techniques of immersion refractometry and interferometry of living cells.

INTRODUCTION

THE number of perfusion chambers which have been designed for the growth and study of tissue cultures since the method was originated by Harrison in 1907 is now very considerable. Initially the purpose of irrigating the explant with fresh medium was to improve the supply of nutritive material and the removal of metabolites, and the early designs of Burrows (1912) and Romeis (1912) were directed solely to that end. Carrell (1931), De Haan (1938), and Lindbergh (1939) introduced numerous ingenious improvements with basically the same purpose. Schade (1933), who was interested primarily in the physico-chemical aspects of tissue culture, provided means for the control not merely of the liquid but also of the gaseous phase of the environment. With this increased interest in the environment of the culture as a study in itself and not merely as an improvement in culture technique, De Haan (1938) and Schade (1933) introduced the first chambers which allowed microscopic observation of the culture during the course of perfusion, although under very unfavourable optical conditions. More recently Hu and others (1951), Christiansen and others (1953), and Schwöbel (1954) have produced designs which provide vastly improved facilities for observation, in particular by phase-contrast microscopy.

The optical requirements may be summarized as follows:

(1) The optical path through all points of the chamber must be constant. This means that the chamber must have plane-parallel walls of uniform thickness and refractive index. In addition the immersion medium must be optically homogeneous, i.e. there must be no concentration gradients. Such gradients are particularly likely to occur when the medium is changed and it is essential to avoid this by thorough irrigation. These conditions are especially

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important in interference microscopy, which is much more easily disturbed by gradients in optical path in the medium, slide, or cover glass than is phase-contrast.

(2) The total thickness should generally not exceed 1 mm. in order to allow focusing of the microscope condenser. This can be increased if a condenser with a long working-distance is available.

(3) The chamber should be capable of being used with a coverslip of standard thickness to meet the optical requirements of the objective.

NEW REQUIREMENTS

The advent of new techniques which are being applied to cells in tissue culture has given rise to some entirely new requirements in a perfusion chamber. The present chamber was designed for the study of tissue cultures by the technique of immersion refractometry (Barer, Ross, and Tkaczyk, 1953; Barer and Joseph, 1954). The new requirements will also apply, however, to much projected biochemical and pharmacological investigation, in which the effect of various compounds on the morphological characters of cells in tissue culture will be tested. Briefly, it is necessary to effect repeated complete changes in the medium in which the culture is immersed, (*a*) in a minimum time, and (*b*) with the use of as small a quantity of fresh medium as possible. This latter requirement may be dictated either by the scarcity or by the expense of some constituent of the medium, e.g. by the expense of the bovine plasma albumin employed in refractometry.

For this purpose none of the perfusion chambers already referred to are suitable. In every case a large reservoir is connected to the culture chamber by a system of tubes and control taps. To change the irrigating medium the reservoir must be emptied and refilled and the new medium run in until the chamber is thoroughly flushed out. As the volume of the connecting tubes and control taps creates a considerable 'dead space' which must be flushed out and refilled in addition to the chamber itself, large demands are made both on time and on the supply of medium if a complete change of medium is to be ensured.

There are three further incidental disadvantages from which previous designs suffer. Though they are almost invariably entitled 'simple' culture chambers, for the most part complicated precision work in either glass, acrylic plastic (Perspex), or even stainless steel is involved in making them. This must make their construction slow and expensive. Secondly, those designs which employ coverslips sealed on to the upper and lower surfaces of the chamber are very liable to leak, since positive pressure is applied to introduce the fresh medium. Great care has to be taken to make the seals strong enough to withstand this hydrostatic pressure, which, although it is small, exerts a considerable thrust on the coverslips owing to their large area. Thirdly, no attention has apparently been directed to the problem of ensuring that the new medium shall sweep out the old with a minimum of

mixing and eddying. These processes must increase the volume needed to ensure complete replacement.

DESIGN AND CONSTRUCTION

The new culture chamber is essentially a modification and simplification of that designed by Christiansen and others (1953). It is constructed from

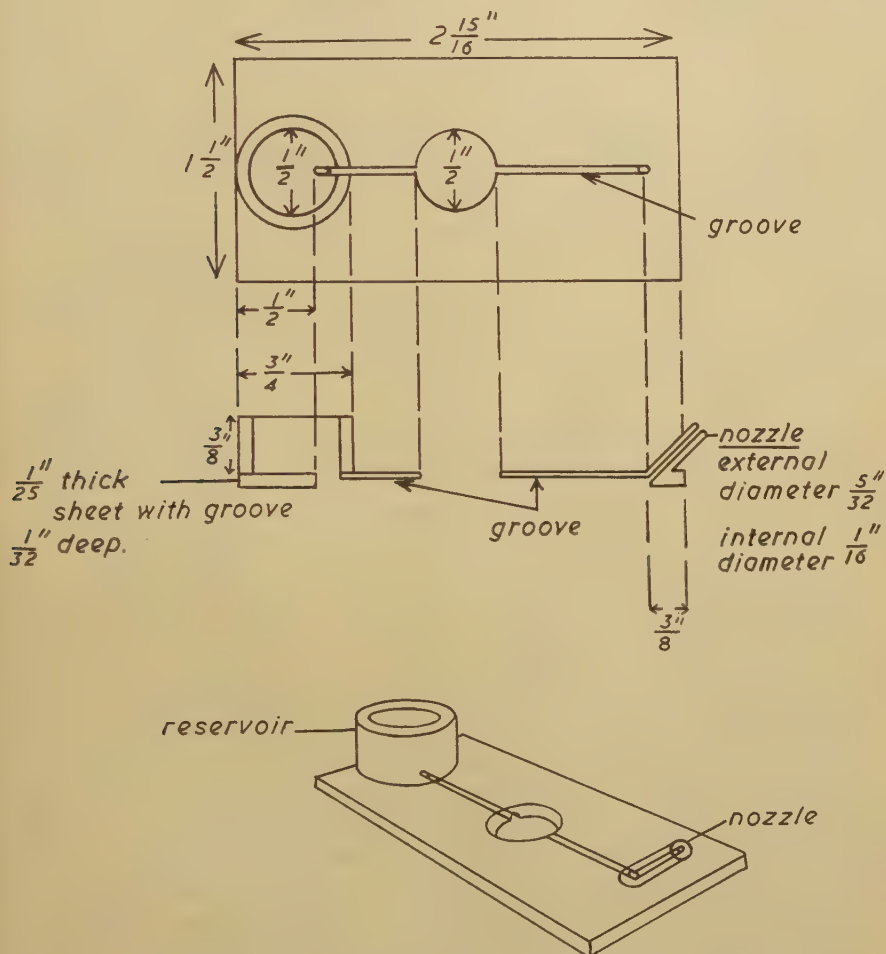


FIG. 1. Plan, elevation, and line drawing of the culture chamber.

acrylic plastic (Perspex) sheet, $\frac{1}{25}$ inch (1 mm.) thick. This is readily obtainable and can be easily worked by a competent carpenter or mechanic without special tools. A high degree of precision is quite unnecessary.

A line drawing, plan, and elevation of the chamber are shown in fig. 1. Provided that the sawing and drilling are done fairly slowly, overheating is not a problem. The two grooves on the under surface on each side of the

control hole are produced by a fine circular saw. At the extreme ends of the grooves holes are drilled through the sheet. The reservoir and nozzle are turned from plastic rod and are attached to the upper surface over the corresponding holes by moistening the opposing surfaces with chloroform. While the chamber has been designed for convenience to the dimensions 3 inches by $1\frac{1}{2}$ inches, it may be reduced without modification to 3 inches by 1 inch size, if for instance it is necessary to fit a warm stage apparatus.

ASSEMBLY AND OPERATION

To set up the chamber, a $2\frac{1}{2}$ inches by $\frac{1}{8}$ inch coverslip is sealed over the lower surface with paraffin wax, covering the central hole and the whole of both grooves leading into it. The coverslip, $\frac{7}{8}$ inch by $\frac{7}{8}$ inch, carrying the

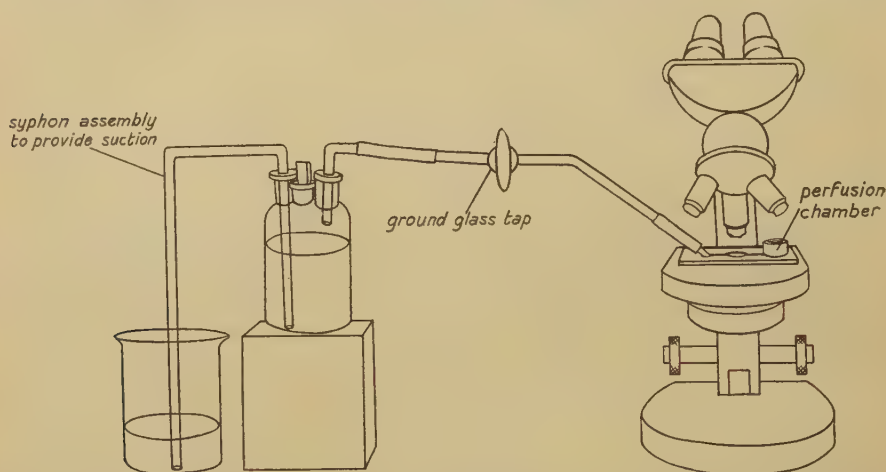


FIG. 2. Sketch of the apparatus. A simple syphon arrangement is used to provide suction.

culture to be examined in a hanging drop, is sealed on to the upper surface over the central hole. No special strength is required in these wax seals. The irrigating medium is run into the reservoir with a Pasteur pipette. A piece of rubber tubing attached to a ground-glass tap leading to a suction apparatus is placed on the nozzle and the medium is sucked into the chamber. The formation of air-bubbles may be avoided by tilting slightly as the fluid runs in. Care must be taken not to empty the reservoir completely with risk of sucking air into the chamber. A simple syphon arrangement as illustrated (fig. 2) or a filter pump may be used to provide suction.

To change the medium in the chamber it is only necessary to suck out the remainder of the old medium from the reservoir with a Pasteur pipette, refill with new medium, and suck it through.

The optical properties of the chamber are excellent, provided that care is taken to seal the coverslips flat on to the surfaces of the chamber.

THE CHAMBER IN USE

It is seen that, apart from its simplicity, the chief innovation in this design is the substitution of negative for positive pressure to effect flow. The medium is sucked into the chamber rather than introduced by gravitational or other force. This has made it possible to eliminate almost entirely the 'dead space' between the reservoir and the chamber. Since the volume of medium needed for complete replacement is thus reduced, the size of the reservoir can also be reduced. This modification has also entirely eliminated the risk of leakage at the paraffin-wax seals, since the suction tends to draw the coverslips against the chamber instead of the reverse.

The time required to replace completely the medium in the chamber depends, first, on the rate of flow of medium, which is directly proportional to the degree of suction applied and inversely proportional to the viscosity of the medium. Secondly, it depends on the amount of mixing which takes place, and this will be discussed below. Even with media of relatively high viscosity, such as concentrated protein or acacia gum solutions, complete replacement can normally be brought about within one minute.

Effects of mixing

The effects of mixing and eddying in the chamber have been studied. In experiments with solutions coloured with methylene blue it was found that solutions of relatively high viscosity (e.g. containing more than 20% of acacia gum) produced the most satisfactory clearance of the old medium. When viscous solutions were interchanged or when a viscous solution replaced one of low viscosity, mixing was negligible and the fresh viscous medium entered the chamber as a separate wave, effecting complete and instantaneous replacement with less than 0.5 ml. of medium. (The volume of the reservoir is 1.2 ml. and that of the chamber 0.13 ml.) With non-viscous solutions (e.g. salines) there was considerable mixing of old and new media. Several designs with two channels leading from the reservoir and entering the chamber at different angles were tried. None of these, however, was found significantly more satisfactory than that with single straight entrance and exit channels. More efficient clearance was produced in this case by creating an intermittent rather than a continuous flow of the fresh medium. When a concentrated aqueous solution of methylene blue was washed out of the chamber with water, it was found that 2 ml. of water were sufficient to reduce the concentration of dye in the chamber (estimated colorimetrically) to between 0.4% and 0.6% of the initial value. Clearance was least satisfactory when a viscous solution was replaced by a non-viscous one. In this case a layer of dense viscous solution persisted on the floor of the chamber. If, however, the chamber was slightly tilted during the change, the viscous layer tended to gravitate towards the exit channel and clearance to less than 0.4% of initial concentration was achieved with 2 ml. of medium. Here also intermittent flow was more efficient in effecting clearance than continuous flow.

Experience with the chamber

The culture chamber has been used in refractometric measurements on chick heart fibroblasts and snail amoebocytes. It is, of course, a condition of its use that the culture or tissue to be examined should adhere firmly to the upper coverslip, whether by natural migration on to it or by attachment by means of a plasma clot. Fig. 3 illustrates an amoebocyte of *Helix aspersa* which has undergone seven successive changes of acacia gum solutions while in the culture chamber. There is no sign of damage, in marked contrast to the appearance produced after a small air-bubble has been in contact with the cell for approximately 15 seconds. This observation must call in question the validity of the interferometric cell-thickness determinations made by Mellors and others (1953), in which the cell was examined when suspended in an air-bubble. Such a cell must have been so grossly damaged as to bear little resemblance to the normal.

The Departmental workshop has so far made one dozen of these culture chambers and they appear to offer no great difficulty in manufacture.

Use under sterile conditions

While the chamber was designed for experiments in which the culture is required to survive only for a few hours and sterility is therefore not essential, it may be easily adapted for use under sterile conditions. The chamber may be sterilized by ultra-violet irradiation (Carlson, Holländer, and Gaulden, 1947) and the reservoir protected from infection between changes of medium by a sterile glass cap. The coverslips may be easily attached so as to preserve the aseptic condition of the chamber. Then, provided that the irrigating fluid is sterile and is introduced into the reservoir with a sterile pipette, complete asepsis is maintained. There is no need to sterilize the suction apparatus.

I am greatly indebted to Dr. R. Barer for his continuous advice and encouragement in the preparation of this paper. Mr. A. B. Alder, F.R.C.S.,

FIG. 3 (plate). Series of photographs showing effect of changing the immersion medium on snail amoebocytes. All photographs were taken with a Winkel-Zeiss semi-apochromatic phase-contrast objective $\times 40$, N.A. 0.75. The heavy absorption of light in the phase plate emphasizes the halo around refractile details.

A, amoebocyte in 0.9% saline.

B, same amoebocyte in 18% neutralized acacia gum solution of the same tonicity as 0.9% saline. The cytoplasm and immersion medium have the same refractive index. The consequent absence of contrast between them makes the cytoplasm 'disappear'.

C, same amoebocyte in 25% neutralized acacia gum solution. The cytoplasm has a lower refractive index than the immersion medium and therefore appears relatively bright ('reversed contrast').

D, same amoebocyte returned to saline. Note that active amoeboid movements have continued. The dark lines at the periphery of the cytoplasm are apparently thickened protoplasmic folds produced during movement.

E, same amoebocyte in saline after four further changes of immersion medium (different concentrations of acacia gum). The cell is still motile.

F, same amoebocyte in saline after an air-bubble 1 mm. in diameter had rested on it for approximately 15 seconds.

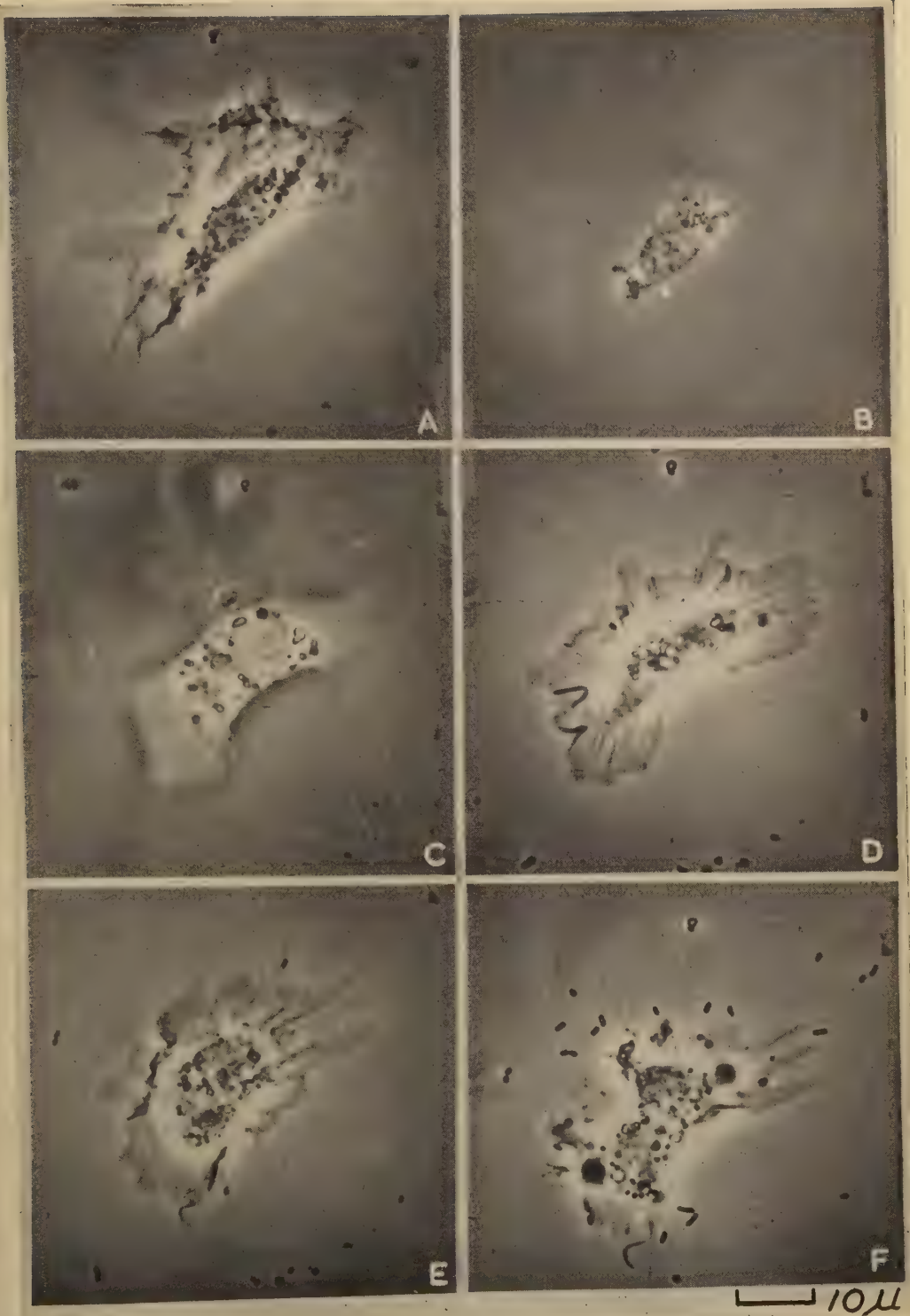


FIG. 3
D. A. T. DICK

made some helpful suggestions. My thanks are due to Mr. P. J. Peade for his skilled execution of the design. This work was carried out while on leave of absence from the Department of Anatomy, University of Glasgow, for which I must thank Prof. G. M. Wyburn.

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A Description of a new Zooplankton Counter

By JAMES WARD

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SUMMARY

The new plankton-counting cell is an annular groove machined in a circular plate of lucite. The plate is mounted on a turntable and driven at a rate of about one revolution in six minutes. Counting is done by observation of the rotating sample as it moves through the field of a microscope. This apparatus provides the convenience of the trough and flow systems currently used and has the advantage of being a more compact unit.

IN the conventional method of phytoplankton enumeration the standard Sedgwick Rafter counting cell has long been used to hold a definite fractional volume of the sample. Cells of this general type modified, particularly with respect to dimensions, have also been commonly used for both total and fractional counts of zooplankton samples. Although these cells are somewhat inconvenient to use a more important criticism is that, even when ruled lines are used to subdivide the area, errors in counting are possible. These may arise through the overlapping of the fields of view used in scanning the cell, and through movement of the cell on the microscope stage which usually disturbs the distribution of the organisms to some extent, decreasing the accuracy of the count. In order to minimize the error, and to increase the convenience of the counting method, a rotating counting cell has been developed which passes the sample slowly under the objective of the microscope with no disturbance to the distribution of organisms. The organisms are maintained in a groove which has the same width as the field of view so that lateral overlap of fields is obviated.

The counting cell proper, as shown in fig. 1, is made of a circular plate of lucite $\frac{1}{2}$ inch in thickness and $6\frac{1}{2}$ inches in diameter. A groove $\frac{3}{16}$ inch deep with sides sloping outwards at an angle of 30 degrees is machined in the upper face near the periphery and the surface of the groove and the outer edge of the plate are polished. The width of the bottom of the groove is slightly less than the diameter of the field of the optical system used in enumeration. In the present apparatus the groove is 4.5 mm. wide for use with $3\times$ objective and $12.5\times$ oculars on a Leitz-Greenough binocular microscope. The slope of the sides of the groove allows complete settling of the organisms and prevents the meniscus from interfering with accurate focusing. Neither the slope of the groove nor dimensions other than the width of the bottom are critical.

The mounting for the cell is the hub and axle of the front wheel of a bicycle. This is firmly bolted in a vertical position to the base plate of the counter. A

lucite pulley 5 inches in diameter is fixed to the upper rim of the hub. The counting cell proper has a concentric recessed depression by which it is centred on this pulley. The cell is easily removed for draining the sample yet quite stable, resting as it does on the face of the 5-inch pulley. The base plate is provided with three levelling screws for adjustment of the counting cell.

An electric timer motor with a speed of one r.p.m. provides the motive

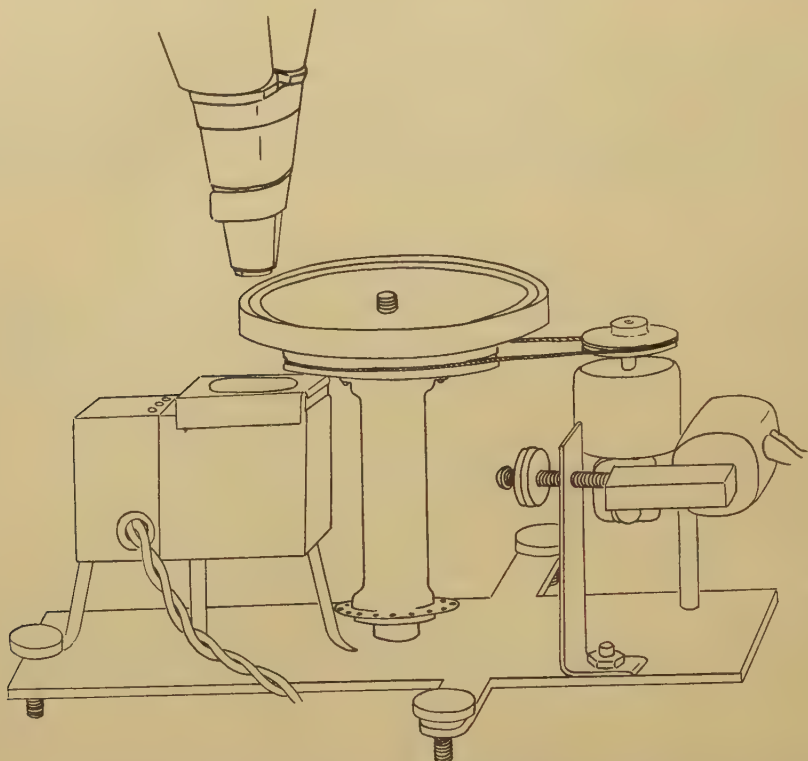


FIG. 1. Side view of counter with working position of microscope objective indicated. The circular counting cell, which rests concentrically on the pulley, is easily removed from the shaft.

power for the counting cell. Suitable drive pulleys are used to give a rate of rotation to the cell of 0.17 r.p.m. and 0.25 r.p.m. respectively. A foot pedal switch is used on the power line to the motor so that the counter can be stopped at will during the count. The motor is hinged to allow adjustment of the tension on the belt when different drive pulleys are used.

The light source is a small microscope lamp fitted with ground glass and blue filter which rests directly under the groove of the cell.

In making total counts of zooplankton most of the supernatant liquid is removed from the sample vial and the organisms washed into the groove of the cell. Sufficient supernatant fluid is used to rinse the vial and to fill the

groove about three-quarters full. The organisms are stirred with a plastic rod to distribute them fairly uniformly about the circumference of the groove and then allowed to settle. The count is begun and terminated at an index mark scratched on the floor of the groove. Evenly spaced transverse scratches about the circumference aid as points of reference as the organisms pass the field of view. When organisms are not too abundant the higher speed of rotation may be used. When more concentrated the slow speed is used and when organisms are clumped the motor can be stopped while individuals in a group are counted. Stopping and starting the cell does not affect the distribution of the organisms in the groove. Counts are recorded on two nine-unit blood counters operated with one hand, the other being left free for focusing. The sample may be removed by pouring it out, this being facilitated by a pouring lip filed in the outer edge of the groove, but it is more convenient to suck the sample up by means of a small lucite pipette with rubber bulb.

Fractional counts may be made by counting the organisms in a definite fraction of the segments marked off in the groove or by counting the organisms in one-half or one-quarter of the circumference. Uneven distribution of the organisms about the circumference and the overlapping of organisms in adjacent segments lead to some error in such counts. Hence when high accuracy is requisite it has been found advisable to subdivide the original sample and count all zooplankters in each sub-sample rather than rely on fractional counts.

This rotating counter has been used by Mrs. E. Jermolajev for some months and has been found to be considerably more convenient than the rectangular cell previously used. The time required to complete a total enumeration of a 10-litre trap sample is reduced and it is believed that the accuracy of the count has also been improved. The apparatus also has the advantage of being more compact than the trough or flow systems used in some laboratories.

Amoeba taylorae n.sp.

By CATHERINE HAYES, S.N.D., B.Sc., Ph.D.

(From the Research Department of Notre Dame College, and the Zoology Department, Glasgow University)

SUMMARY

In material collected from Tannoch Loch, Dunbartonshire, a large free-living, hitherto undescribed amoeba was found. Individual specimens vary in length from 420 to 500 μ and in width from 70 to 140 μ . Viewed over a black background this amoeba looks dense, white, and opaque, while in transmitted light it has a dusky, almost black, hue, due to the presence in the endoplasm of a large number of small crystals uniform in size, slender and pointed at both ends. There is always a uroid at the hinder end surrounded by bits of debris. The one large nucleus is without a karyosome but has masses of chromatin scattered through the nuclear substance. As a rule there is only one large contractile vacuole, but occasionally a few small ones may be seen at the hinder end. A certain number of nutritive spheres are always present. The author considers this amoeba to be a new species and names it *Amoeba taylorae*.

SOURCE OF THE MATERIAL

DURING the summer of 1938, while examining material collected from Tannoch Loch, Dunbartonshire, Scotland, and its outflow stream at Milngavie, I came across a large amoeba which even at a mere glance appeared to differ widely from *A. proteus*, *A. dubia*, *A. villosa*, and *A. nobilis*, all of which had previously been identified from this source by Sister Monica Taylor and all of which were known to me. A large number of *A. villosa*, a considerable number of *A. nobilis*, and a few *A. proteus* were present in the particular lot of material, but there was not the slightest difficulty in distinguishing the new amoeba, for under the low power of the Greenough binocular over a black background it looked dense, white, and opaque in contrast with the somewhat translucent appearance of the other amoebae. When placed on a slide and viewed under an 18 mm. or 4.4 mm. objective it was equally distinct, being of a dusky, almost black, hue. As will be explained later, these appearances are due to the presence in the cytoplasm of a very great number of minute crystals.

Throughout the summer of 1938 a few specimens of the amoeba could be found in every lot of material collected from this particular source. In 1939 I have records of its occurrence in March, April, July, and October, while it was absent from what little material I was able to collect during 1940, 1941, and 1942. In February 1943 I had one specimen from material collected on the 2nd and one from material collected on the 10th. One individual was seen in the spring of 1952.

DESCRIPTION OF THE AMOEBEA

This creature, one of the larger free-living, freshwater amoebae, has often to be freed from adhering debris with a pair of fine needles. If sometimes it

should happen to be spread out more or less fan-like when found, it begins rapidly to assume a very characteristic shape when placed on a slide. The shape referred to is elongated, narrow at the hinder end, and gradually widening out in front into a single broad, flat, directive pseudopodium (fig. 1, A).

The tip of this pseudopodium often bifurcates (fig. 1, B), sometimes re-

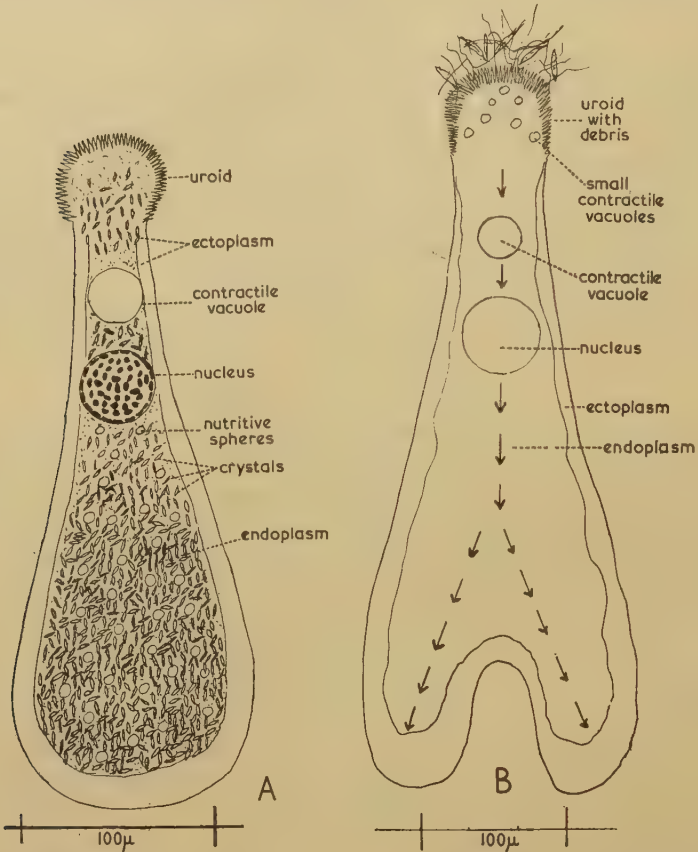


FIG. 1. A, a living *A. taylorae*, showing the uroid, contractile vacuole, nucleus, a great number of small crystals in the endoplasm, and a considerable number of nutritive spheres. B, outline of *A. taylorae*, to show debris attached to the uroid, the bifurcation of the anterior pseudopodium, and the direction (shown by arrows) of the cytoplasmic stream.

maining divided for a considerable time, sometimes returning to the original condition almost at once, while less often the whole of the cytoplasm flows into one limb, the other meantime disappearing, thus restoring the more normal monopodial condition. As a rule there is not more than this one large pseudopodium, there being very little tendency to form lateral pseudopodia.

The average length of the amoeba when it has assumed this very usual shape is about 420μ , though this varies greatly from individual to individual, as I have had specimens measuring nearly 500μ . My records show the width to vary from about 70 to 140μ .

At the hinder end there is always a well-marked uroid (fig. 1, A and B), which never appears elsewhere in this amoeba, a fact differentiating it from *A. villosa* in which a uroid may appear at any point of the ectoplasm. A considerable amount of debris, consisting of small pieces of decayed vegetable matter, long strips of *Oscillatoria*, diatoms, &c., is generally carried with the uroid, and it often looks as if most of this debris consists of the waste indigestible parts of the food (fig. 1, B).

In accordance with the fixed position of the uroid at its posterior end, the amoeba never, so to speak, flows backwards; that is, the posterior end never becomes directive and anterior. When about to change direction the amoeba does so by letting the anterior tip of the pseudopodium flow to one side or the other or by pushing out a large lateral pseudopodium into which all the cytoplasm flows; thus the direction may change through a right angle, but the original uroid persists and is still posterior.

The cytoplasm and its inclusions

The cytoplasm consists of endoplasm and ectoplasm, the latter being very clear and fairly broad, especially at the anterior end where it pours out in wave-like fashion as the creature flows along. The endoplasm is somewhat coarse and granular, but it has a rapid, easy flow, especially down the central region of the body. This is indicated by arrows in fig. 1, B. There are no ectoplasmic folds and therefore the amoeba does not belong to the genus or sub-genus *Chaos*, which is characterized by such folds (Schaeffer, 1926), but to *Metachaos*, as do *A. discoides* and *A. kerrii*. The uroid consists of fine ray-like prolongations of the ectoplasm (fig. 1, A and B).

Embedded in the cytoplasm are:

1. *The contractile vacuole*

There is one large contractile vacuole which occasionally may grow to a great size, 70, 80, or even 90 μ . It is generally situated at the hinder end of the amoeba near the uroid, and is always, even when it is well forward, behind the nucleus. In other large amoebae, e.g. *A. proteus*, *A. discoides*, *A. lescherae*, the contractile vacuole is often alongside, under, overlying, or anterior to the nucleus, as both these structures are carried along in the cytoplasmic stream. The contractile vacuole always bursts near the uroid, and does so very gently; and I have noticed on several occasions that it does not disappear entirely, a very minute droplet remaining to initiate the next vacuole. Although there is only one large contractile vacuole, it often happens that a number of small vacuoles lie beneath the uroid near the large vacuole; these may burst separately and vanish, or one or other of them may increase in size and become the main contractile vacuole. Surrounding the contractile vacuole is a well-defined layer of material of unknown composition and function, which stains slightly with ordinary nuclear dyes. In *A. proteus*, *A. discoides*, and *A. lescherae* (Taylor and Hayes, 1944) this layer is in the form of blocks resembling in appearance the chromatin blocks of the nucleus.

2. The nucleus

The single large nucleus, which is surrounded by a narrow area of clear cytoplasm, has an average diameter of about 30μ , though individual nuclei may be somewhat less or considerably greater in diameter (fig. 2, A and B). Its shape seems to be that of a biconvex lens and hence it appears circular when viewed from above and elliptical when seen in side view.

The ground-substance of the nucleus is coarse and gives the impression of being viscid and stiff, while the chromatin masses are large and conspicuous and, as there is no karyosome present, they are scattered evenly through the nuclear substance, only occasionally appearing as a definite layer underlying

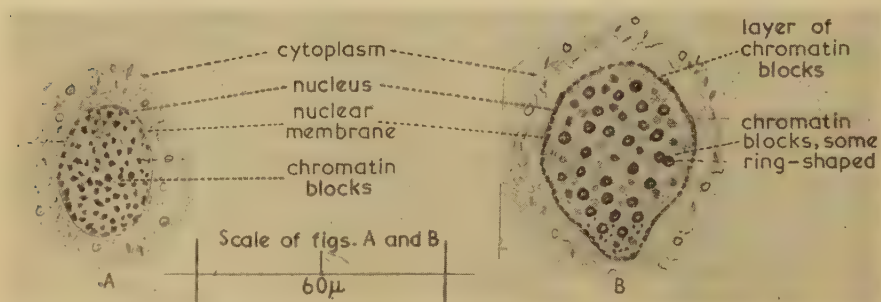


FIG. 2. A, nucleus of ordinary size, with very thin nuclear membrane and chromatin blocks scattered throughout the nuclear substance. B, a very large nucleus with only a trace of the nuclear membrane, but with a distinct layer of chromatin blocks round the periphery. Fig. 2 was drawn from specimens fixed in Bouin's fluid and stained in Ehrlich's haematoxylin and light green. A Zeiss apochromat 2 mm. oil immersion objective was used.

the nuclear membrane (figs. 1, A, and 2, B). Fixed and stained preparations reveal the nuclear membrane to be thin (fig. 2, A). The nucleus is often observed at the posterior end of the creature, whence it may move slowly forward to about the middle of the body; here it generally remains stationary while the cytoplasm flows past it rapidly, thus restoring it to its original posterior position. The slow movement of the nucleus through the rapid cytoplasmic stream and its ability to remain at rest in this stream is indicative of its viscid and rather solid nature. The nuclei of *A. proteus*, *A. discoides* (Hayes, 1938), *A. lescherae* (Taylor and Hayes, 1944), and *A. kerrii* (Taylor, 1947), which appear to be more fluid, are carried along rapidly in the cytoplasmic stream and rarely remain stationary.

I have not been able to procure a sufficient number of specimens to work out the nuclear division.

3. Nutritive spheres

A certain number of nutritive spheres are always present (fig. 1, A). Generally these are small, but in two or three individuals examined the spheres were large and greenish in colour.

4. Crystals

The most striking feature of this amoeba is the very great number of small crystals which it normally contains (fig. 1, A). As already mentioned, it is the presence of these crystals that causes the amoeba to appear opaque in reflected light and dark in transmitted light. Most of the crystals are slender and sharply pointed at both ends, but the points and edges of many of them are rounded so that they appear elliptical. As in the other amoebae, the crystals dissolve in the process of fixing and dehydrating.

Food of the amoeba

As far as I have been able to ascertain, this amoeba is not a diatom-feeder, though the material from which I have obtained it is exceedingly rich in many forms of diatoms and swarms with *A. villosa*, a heavy diatom-feeder; it is often possible to count as many as 20 diatoms (of various varieties) in the cytoplasm of one individual. Flagellates and ciliates seem to be the principal food organisms. Occasionally, however, especially in the early part of the year, the amoeba's numerous food-vacuoles contain spherical bodies, golden brown in colour. This is also true of *A. nobilis* and *A. villosa*, and especially *A. hugonis*. After it was ascertained that these bodies were not encysted spores, the material was submitted to Professor E. G. Pringsheim, who identified the spherules for Sister Monica Taylor as consisting of a very hard core of a chemical substance that he could not identify, covered with a layer of carotene. Pringsheim and Horasse (1950) show that masses of pigment of varying sizes having the same composition as the eye-spot occur in a variety of *E. gracilis*. Pringsheim expressed the view that the cultures containing *Euglena* thus affected were not in a healthy condition. The question arises, how do the amoebae obtain these spherical bodies? Do they ingest the *E. gracilis*, partly digest it, and get rid of the carotene bodies by defaecation? Sister Monica Taylor (1952) could not decide the question for *A. hugonis*. She never found the golden spherules apart from the living animal.

The fact of its not being a diatom-feeder is beneficial from the point of view of bringing the amoeba under laboratory culture conditions, as flagellate and ciliate food is much more easily produced all the year round in the laboratory than are diatoms.

Life-history

Not finding the amoeba in large numbers has prevented me from collecting any facts about its life-history, but I have had two or three small amoebae which might very well be its young stage. I hope to evolve a technique for keeping the amoeba under laboratory conditions and so be able to elucidate its life-history.

DISCUSSION ON THE IDENTIFICATION OF THIS AMOEBEA

When consulting the literature with a view to the identification of the amoeba just described I was impressed by the resemblance between it and

an amoeba identified in Kansas by M. Anthony Payne (1930) as *A. granulosa*. A sub-title to her paper records the fact that this is the third record of the appearance of *A. granulosa*, i.e. 'Grüber's Rare Ameba'. Grüber's record was made in 1885, and he gave the name to a small amoeba ('von ungefähr 0,03 mm. Durchmesser'), of which he gives very few details. The second record of this amoeba was that of Penard in 1902. A glance at Grüber's figure was sufficient to convince me that the amoeba which I have described is not Grüber's 'Rare Ameba'. Penard's (1902) account is much fuller than Grüber's (indeed, I fail to see how Penard could have thought that the amoeba he described was identical with Grüber's *A. granulosa*), and many points, such as the size, the character of the crystals, and the general appearance of the amoeba caused by these crystals are in accordance with my description, but he states that his amoeba has a spherical nucleus and large pseudopodia in different parts of the body, while he makes no mention of the presence of a uroid. Payne's specimens were smaller than Penard's, yet she agrees with him on other details except that she says that few lateral pseudopodia are formed during locomotion and emphatically adds that a 'uroid is not present and the ameoba never carries debris'.

Both these authors figure their amoeba and, while the general appearance and in particular the anterior portion of Payne's fig. 5 does resemble my amoeba, the posterior portion of all her figures and the whole of Penard's figure differ from my findings. Cash (1905) describes an amoeba from Hale Moss, Cheshire, which he calls *A. proteus* var. *granulosa*, but from his figures it is quite evident that my amoeba bears little resemblance to it and even less to Leidy's (1879) fig. 4, pl. I, which Cash considers to be the same species as his. Payne considers that Cash's figures were drawn from *A. proteus* or *A. discoides* and in this I agree with her, especially in view of the fact that under certain physiological conditions both these amoebae may be very much packed with crystals. The 'mulberry-shaped caudal extremity', which Cash refers to and figures, is not a uroid but a temporary condition of the ectoplasm, such as may often be seen in all the large amoebae with which I am acquainted.

To sum up, although resembling *A. granulosa* in some respects, the amoeba described in this paper differs from it in having a biconvex nucleus, a permanent posterior uroid, and a great tendency to the monopodial condition when creeping. In view of these outstanding characteristics and believing that it has not hitherto been recorded, I propose to call the present species *Amoeba taylorae*, in dedication to Sister Monica Taylor, who has devoted the research of her lifetime to the genus *Amoeba*, its species, habits, and culture.

DIAGNOSIS

This amoeba was found in a freshwater loch to which there was a constant inflow of clean water and a similar outflow. The loch was also well stocked with various kinds of water weeds. In size the amoeba may reach 500μ long by 140μ at the broadest part. The shape is generally elongated and monopodial,

the anterior end being broader than the rest of the body. The posterior end always possesses a uroid which generally has debris attached to it. The ectoplasm is fairly voluminous, especially at the anterior tip. The endoplasm is packed with small, slender-pointed crystals and hence is very dark in colour. There is one large contractile vacuole, which generally occupies a posterior position; occasionally there may be a few small vacuoles near the uroid. The one large nucleus (about 30μ in diameter) is biconvex; it has no karyosome but blocks of chromatin are scattered through it. Sometimes a layer of chromatin blocks lines the thin nuclear membrane. There are no longitudinal folds in the ectoplasm; in this it resembles *A. discoides* and *A. kerrii* and differs from *A. proteus* and *A. lescherae*. It belongs to Schaeffer's group *Metachaos*. I consider it a new species and name it *Amoeba taylorae*.

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Adaptation to Rock Boring in *Botula* and *Lithophaga* (Lamellibranchia, Mytilidae) with a Discussion on the Evolution of this Habit

By C. M. YONGE

(From the University of Glasgow)

SUMMARY

All species of *Botula* and of *Lithophaga* live within borings they excavate in rock, In *Botula* boring is mechanical, into soft non-calcareous rocks. Firm attachment is made by byssal threads arranged in a large anterior and a small posterior group. The rock is abraded by the dorsal surfaces of the valves, which are deeply eroded; the forces responsible are contraction of the byssal and pedal retractors and the powerful opening thrust of the long ligament. There is no rotation within the boring. In *Lithophaga* boring is confined to calcareous rocks, byssal attachment being weak and the valves little eroded. The fused inner lobes of the mantle edges may protrude from between the valves anteriorly. They are applied to the head of the boring and it is postulated that they secrete an acid mucus. Some species of *Lithophaga* bore straight into rock, others rotate in the boring.

Aggregations of foreign particles on the valves of *Botula* and *Lithophaga* are due to the effect of accumulations of mucous glands both anterior and posterior to the ligament. The origin and function of these glandular masses is discussed by reference to conditions in the allied *Modiolus*. They help in disposing of sediment. In *Lithophaga* the anterior glands, at least, probably secrete an acid mucus. Crystalline calcareous secretions which adventitiously lengthen the shells of certain species of *Lithophaga* are produced by the walls of the siphons, which may also lay down a calcareous lining to the boring.

Evolution of the boring habit in the Mytilidae is discussed in relation to shell-form. The elongate shell of the dimyarian types *Botula* and *Lithophaga* is compared with those of *Ensis* or *Tagelus* and shown to be derived from that of a heteromyarian ancestor not unlike *Modiolus*. The effect of the tangential component in the growth of the mantle/shell counteracts that of reduction of the anterior end of the body and of the mantle/shell associated with heteromyarianism and is also concerned with lengthening of the ligament. Change in form in the Mytilidae made possible change in habit.

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INTRODUCTION

A VARIETY of unrelated Lamellibranchia have become adapted for boring into rock. As suggested elsewhere (Yonge, 1951a), evolution of this habit would seem to have proceeded along one of two lines. It may have done so by further specialization of species already adapted for deep-burrowing in soft substrata, enabling them to penetrate stiff clay and finally rock. Examples are provided by the Pholads generally and also by *Platyodon* among the Myidae (Yonge, 1951b). The alternative route may have been by way of the 'nestling' habit possessed by various bivalves that live attached by byssus threads within depressions and crevices among rocks. Initially they may enlarge the depressions and eventually become active borers into the rock. Such animals are exemplified by species of *Hiatella* (*Saxicava*) described by Hunter (1949), and by *Tridacna crocea* among the Tridacnidae (Yonge, 1936).

Also to be included within the latter category are species of the Mytilidae, the best known being *Lithophaga* (*Lithodomus*) *lithophaga*, the date mussel of the Mediterranean. Like all species of this genus (or subgenus according to Thiele (1935)), *L. lithophaga* bores only into calcareous rock, doing so, it has been claimed, by the aid of an acid secretion from the mantle (Carazzi, 1892, 1903; List, 1902; Otter, 1937; Kühnelt, 1930). Details of the procedure employed in boring have hitherto been somewhat obscure.

Thiele (1935) subdivides the genus *Lithophaga* into subgenera of which *Lithophaga* s.s., further split by him into three sections, is one and *Botula* is another. Three species of the latter, according to Keen (1937), occur along the Pacific coast of North America. Two of these, *B. falcata* and *B. californiensis*, were collected between tide-marks near San Francisco. They proved of particular interest because although they bore they do so mechanically. Acid secretion would in any case be useless because they occur in non-calcareous rocks. Examination of these animals proved of unusual interest in view both of the absence of previous work and of the light thrown on the evolution of the boring habit in the genus *Lithophaga*.

The observations on *Botula* spp. were conducted in the Department of Zoology, University of California, Berkeley, while the author was Visiting Professor in the spring of 1949. The further work on *Lithophaga plumula* was carried out later that year at the Hopkins Marine Station, Pacific Grove. Some observations on other species of *Lithophaga* were subsequently made at

Bermuda in 1951. Thanks are here rendered to the late Professor H. Kirby and members of the staff at Berkeley, in particular Dr. R. I. Smith, and also to Dr. R. Boline, Acting Director at Pacific Grove. At Glasgow important help has been given by Dr. Vera Warrender, who made the drawings reproduced in figs. 1 and 8, and by Dr. H. F. Steedman and Mr. G. Owen, who prepared sections.

OBSERVATIONS ON *BOTULA*

Botula (Adula) falcata (Gould) and *B. (A.) californiensis* (Philippi), respectively the hooked pea-pod shell and the Californian pea-pod shell (Keep and Baily, 1935), are true rock-borers. As shown in fig. 1, they differ markedly

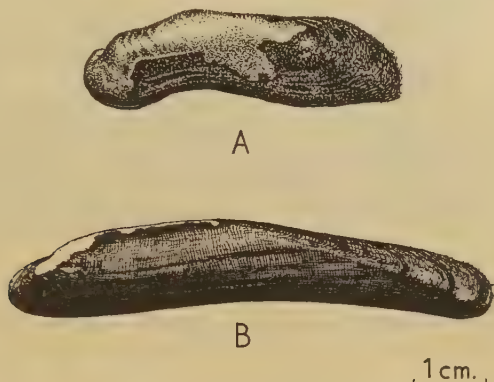


FIG. 1. A, *Botula californiensis*; B, *B. falcata*, both viewed from left side, showing erosion of shell dorsally and accumulation of material on posterior region of valves.

in form. Both are long, but especially *B. falcata*, which is also straight, *B. californiensis* being markedly concave ventrally. The valves of this species are more angular and the umbones more pronounced. Apart from the form, the thick periostracum also differs in the two species: that of *B. falcata* is thrown into numerous fine transverse ridges, which are absent in the other species, where the periostracum is rather darker in colour. In adult specimens of either species the periostracum is always eroded over a wide area dorsally, an important matter which will be discussed later. In both species the posterior region of the valves is covered with an amorphous encrustation.

In the regions where specimens were collected *B. falcata* was much the commoner, and laboratory observations were confined to this species. But, at any rate between tide-marks, the two species have the same habits and bore into the same type of rock. Smith and Gordon (1948) report that both occur in Monterey Bay, boring into shale, down to depths of 15 fathoms. Here also *B. falcata* is much the more numerous. *B. californiensis* extends farther north, ranging between latitude 33° and 49° , whereas *B. falcata* occurs between 33° and 43° (Keen, 1937). It is possible, therefore, that the two species, although they overlap so much in distribution, do have different breeding-temperatures. Some difference in habitat may be revealed by further field observations.

(a) *Habitat*. *B. falcata*, with occasional specimens of the other species, was collected in quantity at Moss Beach and Frenchman's Reef to the south and at Duxbury Reef to the north of San Francisco. Specimens varied in length from a maximum of 8.5 cm. to a minimum of about 1.5 cm. The latter may represent the size at one year, because the larger individuals were all spawning in May when most of the collections were made. All specimens were restricted to bands of soft mudstone, also bored into by *Platyodon cancellatus* as described elsewhere (Yonge, 1951b), in middle and upper tidal levels. The harder but also non-calcareous rocks farther down the shore contained other borers, notably species of *Pholadidea*.

In most cases the animals occupied borings excavated by them. The openings were small and difficult to detect unless the siphonal extensions of the

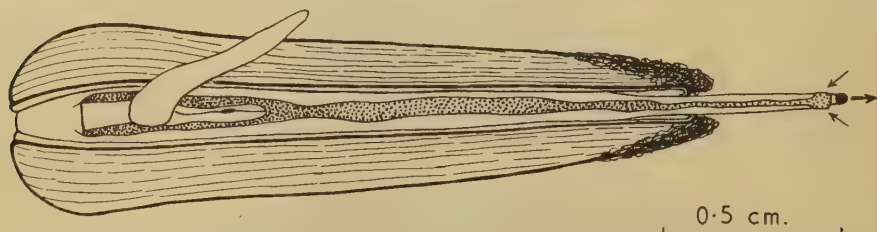


FIG. 2. *B. falcata*, ventral view of animal removed from boring with foot and siphons extended. Note extent of separation of mantle lobes ventrally (fusion of inner lobes of mantle edge only anteriorly) and, again, accumulations of material on posterior surface of valves.

mantle were projecting through them, as they usually were when under water and even sometimes when exposed if the surrounding rock surface was damp. Occasionally animals were found in pre-existing borings and not always extending to the base of these. In these circumstances, the animals undoubtedly move deeper as they grow, the posterior end of the shell with the siphonal extensions retaining a constant position in relation to the surface of the rock. It is highly probable that, after settlement from the plankton, the young animals make initial byssal attachment in crevices or, occasionally, within old borings. They may be presumed to start active boring as soon as growth brings the anterior end of the shell in contact with the rock surface. The means whereby they reach final settlement on appropriately soft rock remain to be discovered. For detailed examination, animals were usually collected by breaking off pieces of rock in which they were boring, from which they were later removed in the laboratory.

(b) *Observations on intact animals*. In certain important respects *Botula* is intermediate in structure between *Modiolus* and *Lithophaga*. In the former, as in the well-known *Mytilus*, the mantle tissues nowhere project significantly beyond the margin of the valves even when these are widely separated. There is no posterior siphonal extension to the mantle edge. *Botula* resembles *Modiolus* and differs from *Lithophaga* in the absence of any anterior protrusion of the mantle margins, but it resembles the latter in the possession of long posterior siphonal extensions formed exclusively from the inner lobes of the

mantle edges. Their form will be described later when dealing with those of *Lithophaga*. Here, as in all the Mytilidae, the exhalant tube is complete, the inner lobes of the mantle edges fusing ventrally, but the ventral boundary of the inhalant aperture is formed by local apposition of the mantle surfaces with the formation of a functional but not a structural tube (figs. 2 and 3). The siphons so formed are capable of great extension. In an animal of shell-length 5.7 cm., maximum siphonal extension of 2.7 cm. was noted, the siphons curling up and then bending forward along the dorsal surface of the shell. This, however, was exceptional; the siphons do not normally extend for lengths greater than one-quarter of the shell. Their tips are speckled with brown pig-



FIG. 3. *B. falcata*, boring opened to expose dorsal side of contained animal. The siphons are extended and the animal withdrawn from head of boring. Erosion of shell dorsally and posterior accumulations also shown.

ment to a varying extent probably connected with the degree of exposure to light.

The foot, situated near the anterior end, is long and thin (fig. 2). In life it can extend forward or back between the shell and the wall of the boring. Once the animal is established in its boring the only, but all-important, function of the foot is to plant the byssus threads which issue from the aperture of the byssus gland (fig. 6) and are secured to the 'floor' of the boring.

The only other external features demanding attention are the abraded areas of the shell around the umbones (shown in figs. 1, 3, and 4), and the accumulation of material which adheres to the posterior region of the shell, and these are discussed later. Within the mantle cavity the disposition of the organs is similar to that in other Mytilidae, including *Lithophaga*, of which some account will be given.

(c) *Behaviour within boring.* If a boring be opened so as to expose the dorsal surface of the shell, as shown in fig. 3, the animal resumes normal behaviour when replaced in sea-water. Much can then be learnt about the method of boring. When the siphons are extended, as shown in fig. 3, the shell is withdrawn somewhat from the head of the boring, to a distance of some 2 mm. in an animal 2 cm. long. The space so left is usually occupied by fragments of rock debris mixed with mucus. In the intact boring this is probably quickly cleared away with other waste by way of the inhalant aperture. When the siphons are touched they immediately withdraw, the valves at least partially close, while

the shell moves forward so that the anterior end is pressed against the head of the boring. After a short time the shell reopens, the siphons extend, and the animal moves away from the head of the boring.

Three sets of muscles have to do with these movements. The siphons are pulled back by their retractors, the two adductors close the shell, and the larger posterior and anterior byssal retractors (fig. 6, A) pull the animal forward and then back respectively. Thus the normal reaction to stimulation is withdrawal as far as possible into the protection of the boring, a possible indication of the manner in which the boring habit originated.

The animals remain capable of life outside a boring. If removed, they reattach themselves by byssus threads and may also climb up the sides of glass vessels, precisely as do young *Mytilus*.

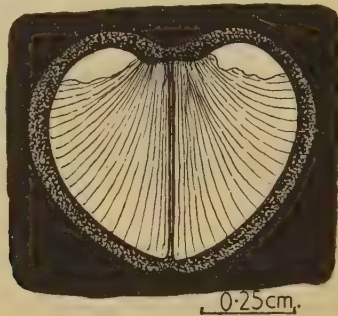


FIG. 4. *B. falcata*, boring opened at head end to expose anterior end of borer. Characteristic cross-sectional outline of shell reflected in that of the boring.

(d) *Form of the boring*. As shown in fig. 4, which represents the head of a boring opened with the animal *in situ* but somewhat withdrawn, the boring is not circular in cross-section but is moulded by the form of the shell. There is a pronounced 'dorsal' ridge which occupies the region between the umbones and a much less pronounced 'ventral' ridge, also indicated in fig. 3, between the free margins of the valves. Conditions thus resemble those already described in *Platyodon cancellatus* (Yonge, 1951b), a eulamellibranch bivalve which resembles *Botula* only in habit, boring into the same soft rocks. Both animals must grind their way straight into this. The presence of the ridges shows that they do not revolve, turning first in the one direction and then in the other, as do the Pholads which cut a boring which is circular in cross-section.

(e) *Method of boring*. This is purely mechanical; the rock is not calcareous, acid has no effect upon it. Actual abrasion of the rock must almost exclusively be carried out by the dorsal surfaces of the valves, along a broad region running forward and backward from the umbones. There, as shown in figs. 1 and 3, the thick periostracum is worn away and the underlying calcareous layer deeply eroded. The forces responsible for boring are the opening thrust of the ligament and the contraction of the byssal and pedal retractors. The ligament—to be fully discussed later—because it is straight is exceptionally long and with a correspondingly powerful opening thrust. If a valve is cracked between

the ligament and either of the adductors, the portion attached to the ligament is usually pulled away from the underlying tissues, so great is the opening thrust. Thus, when the adductors relax, the valves will be pressed against the walls of the boring and then scraped forward or back along these when the byssal and pedal retractors contract.

Firm attachment is needed for boring, and this is provided by the very numerous byssal threads. After removal of the animal, the disposition of these on the 'ventral' side of the boring is revealed. As shown in fig. 5, they consist of an anterior and a posterior group. The former are attached in a semicircle some 5 mm. short of the head of the boring (animal of shell-length 2.1 cm.)

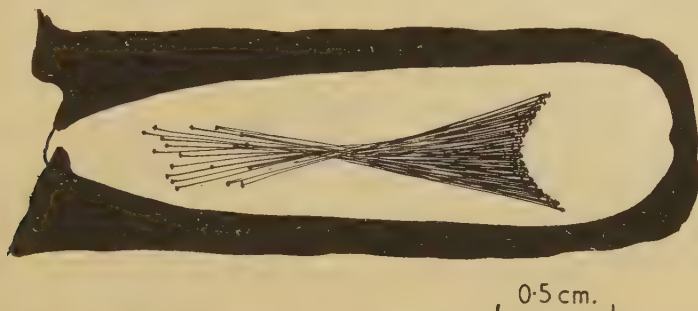


FIG. 5. *B. falcata*, boring opened to display attachment to 'floor' of byssus threads in large anterior and small posterior groups.

and are some four times as numerous as those forming the posterior group, which are irregularly arranged. The first group of threads must be planted when the foot is extended far forward, the second group when it is turned back. The final effect is to anchor the animal most securely within the boring.

Comparison with conditions in *Mytilus* or *Modiolus* is interesting. In species of these genera the less numerous byssal threads are disposed in a rough circle around the base of the foot, enabling the animal to resist wave action from any direction. In *Botula*, where movement is restricted to the line of the boring, there are no laterally attached threads. Moreover, of the two possible movements, forward and back, the former involves, as the animal grows, pressure against the rock with consequent extension of the boring. There is much less resistance when the animal moves back in the boring, although abrasion of the shell posterior to the umbones and the relatively powerful anterior byssal retractor (fig. 6, A) indicate that the boring is widened when the shell is drawn back. Nevertheless, the difference in numbers between the anterior and posterior groups of byssal threads is doubtless a sound indication of the firmness of attachment and so of the amount of work done in forward and backward movement respectively. Discussion on the origin of the boring habit is reserved until later.

(f) *Material adherent to the shell.* The nature and origin of the material which adheres to the postero-dorsal regions of the valves remains to be discussed. As shown in figs. 2 and 3, this surface of the valves is covered with an

encrustation containing fragments of sand or rock and, especially in older animals, many byssus threads. The posterior end of the valves may even be somewhat extended adventitiously. Unlike *Lithophaga*, the matrix is non-calcareous and is probably mucoid. As will be shown, after description of

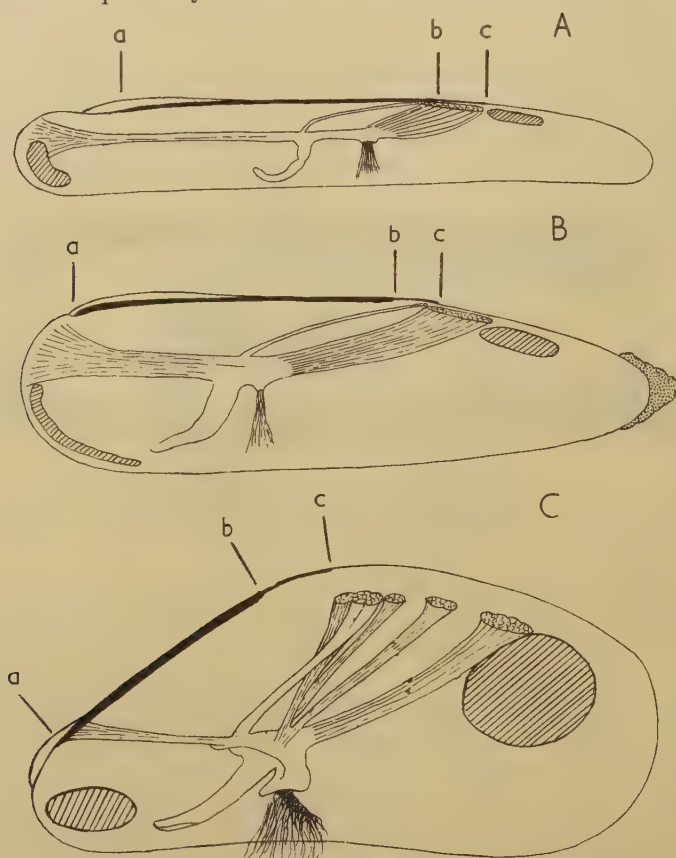


FIG. 6. Comparison of arrangement of anterior and posterior adductors, foot and byssus, with anterior and posterior pedal and byssal retractors, and ligament in: A, *Botula falcata*; B, *Lithophaga plumula*; C, *Modiolus modiolus* (all drawn the same length). *a-b* represents extent of 'true' ligament, *b-c* posterior extension formed by periostracum only (see fig. 15). Calcareous posterior extension of shell shown in *L. plumula*.

conditions in *Lithophaga*, there is good evidence that this matrix is derived from the secretion of mucous glands in the mantle margins between the posterior end of the ligament and the base of the siphons.

OBSERVATIONS ON *LITHOPHAGA PLUMULA*

Lithophaga plumula is typical of the genus in that it bores exclusively into calcareous rocks, including the shells of other bivalves. It occurs commonly off the coast of California, the centre of its distribution being probably well to the north. MacGinitie and MacGinitie (1949) note that near Los Angeles

it spawns in January, i.e. is probably there near the southern end of its range. The form of the shell is shown in fig. 7. It is more massive, shorter in relation to breadth and height, than either species of *Botula*, and the shell is thicker. When viewed from the anterior end the shell appears almost circular in cross-section; the umbones do not project as they do in *Botula*. Posteriorly the valves



FIG. 7. *Lithophaga plumula*, viewed from left side, showing amorphous calcareous concretions covering periostracum generally and somewhat plumose crystalline encrustation at the posterior end.

are somewhat compressed laterally and they are pointed. There is little evidence of abrasion except in large specimens, and even in these the periostracum is never perforated. This is light brown in colour and is largely covered with encrustations which, as noted by Kühnelt (1930) in other species, are of two types, which he terms non-crystalline and crystalline. The former consists

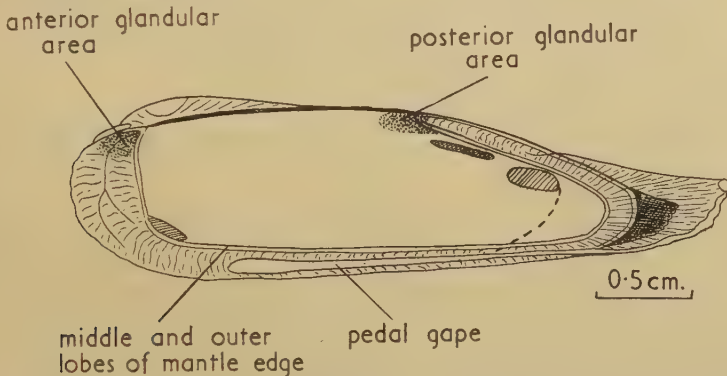


FIG. 8. *L. plumula*, left valve removed, showing extent of inner lobes of mantle edge forming siphons posteriorly and capable also of extrusion where fused anteriorly. Broken line indicates attachment posteriorly of inner mantle lobes to mantle. Position of glandular areas in relation to mantle lobes also shown.

of a thin, somewhat granular layer which covers much of the surface of the shell although varying greatly in amount and in distribution from specimen to specimen. The other encrustation is thick and rugose; it covers the postero-dorsal region of the shell only and projects for some distance beyond the posterior margin of the valves, as shown in figs. 8, 9, and 10. Both types of encrustation are calcareous. Fuller description is given later.

(a) *Habitat*. Specimens varying in length from 1.8 to about 6 cm. and in maximum depth (and breadth) from 0.5 to 1.5 cm. were obtained from shallow depths in Monterey Bay, where they occur boring into calcareous shale. The animals lived well in running sea-water in the laboratory, where observations

were made upon them both *in situ* and after removal from the borings. It proved much easier to make detailed study of the living animal than it was in *Botula*, but the two differ only in minor details.

(b) *Mantle and siphons*. The nature of the mantle edge is important. As shown most clearly in fig. 10, the outer (secretory) lobe of the mantle edge everywhere follows the margin of the shell, to which it is firmly connected by means of the thick periostracum which arises between the outer and middle lobes. The latter consists of a thin ridge which also follows closely the margin of the valves. The significant lobe is the inner (muscular) one (the so-called velum or pallial curtain) which forms a broad projecting fold along the ventral surface. These lobes on the two sides fuse anteriorly—for a length of 1.2 cm. in an animal with a shell 4.5 cm. long—the point of fusion constituting the anterior margin of the pedal gape (fig. 9). These fused tissues can be pro-

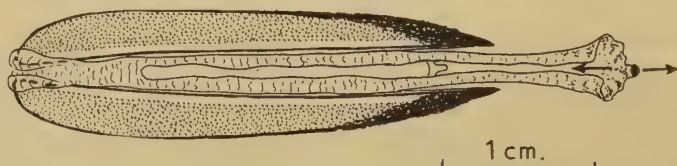


FIG. 9. *L. plumula*, ventral view of animal removed from boring, with siphon extended and full length of pedal gape shown with extruded anterior pallial tissues. Broken lines indicate anterior margins of valves. Amorphous and crystalline calcareous encrustations on shell shown.

truded for some distance around the anterior and antero-ventral margins of the valves, as shown in figs. 8 and 9. Such protrusion of anterior pallial tissues does *not* occur in *Botula*; it will be shown to represent a most important adaptation in *Lithophaga*.

Posteriorly, these inner mantle lobes are extended to form the siphons (fig. 8), the exhalant tube alone being complete. At the base of the fusion of the lobes which constitutes the partition between the two siphonal openings there is a valvular siphonal membrane (figs. 9 and 10). Kellogg (1915) has described similar structures in *Mytilus* spp., while they also occur in members of the Mactracea, e.g. species of *Spisula* and *Schizothaerus* (Kellogg, 1915; Yonge, 1948). The membrane is directed back when the valves are open (fig. 10) but is pulled forward so that it assumes a vertical position when the shell closes. Around the posterior end, as shown in figs. 9 and 10, the inner lobes of the mantle edge are separated by a wide siphonal embayment from the middle and outer lobes, which retain close connexion with the margin of the shell. The siphonal extensions formed by the inner mantle tissues can thus be withdrawn within the shell when the siphonal retractors contract. Conditions are similar in *Botula*, the siphons in both genera representing great posterior enlargements of the inner lobes with consequent wide separation from the middle and outer lobes. In *Modiolus* and *Mytilus* these inner mantle lobes do little more than fringe the inhalant and exhalant apertures.

Dorsal to the exhalant siphon the inner mantle lobes fuse, forming a layer of tissue which extends from the base of the siphons to the region where the

middle lobe and the periostracal groove bend round from one valve to the other at the posterior end of the ligament (see fig. 15). This posterior region of inner mantle lobe fusion projects from between the valves when the siphons are extended (fig. 8). Similar protrusion of mantle tissues occurs in *Botula*, and the significance in both cases will be discussed later.

(c) *Ciliary currents*. Opportunity was taken when examining the mantle cavity in living animals to follow the course of the currents. Those on the ctenidia are similar to the currents described in *Mytilus* by Orton (1912) and

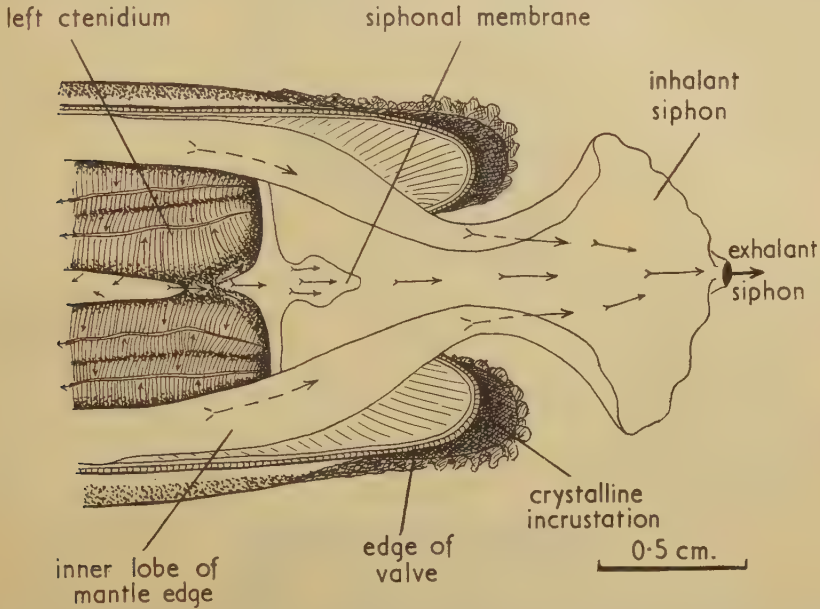


FIG. 10. *L. plumula*, posterior end of mantle cavity viewed, in life, from ventral aspect, with siphons partly expanded and mantle margins separated. Arrows indicate direction of ciliary currents; feathered arrows, cleansing currents (where broken on unexposed surfaces).

Kellogg (1915). Owing to the absence of ventral fusion at the base of the inhalant siphon, pseudofaeces and other waste is carried posteriorly (see feathered arrows in fig. 10) along the inner margin of the inner mantle lobes, the two streams converging in the mid-line dorsally, i.e. at the tip of the inhalant siphon, as shown in fig. 10. As suggested by Kellogg (1915) in the case of *Mytilus*, the siphonal membrane probably serves to 'shield the waste materials from the broad incoming stream of water'. It is ciliated, particles being carried to the tip, i.e. posteriorly when it is in its usual position. In *Lithophaga* (and *Botula*) the inhalant current will pour inward on either side of this membrane, from the under (or anterior) surface of which waste material will be passed on to the dorsal surface of the inhalant siphon for eventual extrusion.

(d) *Behaviour within the boring*. It is more difficult to open the borings without affecting the animal than it is with *Botula*, but this was done success-

fully on a few occasions, the animal continuing to function normally. As in *Botula*, when the siphons are withdrawn the anterior end of the animal is simultaneously pulled back from the end of the boring. An animal of shell length 5.0 cm. drew back in this way for a distance of 9 mm. In certain cases,



FIG. 11. *L. plumula*, boring opened to show dorsal surface of anterior end of shell with anterior pallial tissues protruded against head of boring. Arrows show direction of ciliary currents.

two of which are illustrated in figs. 11 and 12, what appeared to be the normal behaviour was observed. The siphons were fully distended while anteriorly the fused inner mantle lobes were extruded, either symmetrically or on the one side only (figs. 11 and 12 respectively).



FIG. 12. *L. plumula*, animal *in situ* in boring, anterior pallial tissues extruded on left side only. Broken line indicates anterior margin of left valve.

Carazzi (1892, 1903), List (1902), Pelseneer (1911), and Kühnelt (1930) all considered that this region of the mantle must be responsible for secretion of acid and so for dissolution of the calcareous rock into which all species of *Lithophaga* exclusively bore. But actual protrusion and application of these tissues to the head of the boring have never previously been observed *in situ*. When the shell withdraws, much finely granular material embedded in mucus

is exposed, especially where these mantle tissues have been applied to the head of the boring. As shown in fig. 11, the protruded surface is ciliated and currents carry particles with mucus ventrally and then posteriorly. In all Mytilacea examined (e.g. *Modiolus modiolus*), this is the route by which material is conveyed from this region into the mantle cavity (entering it through the pedal gape). Thence it will be conducted, by the route already indicated, to the dorsal side of the inhalant siphon for extrusion.

(e) *Nature of the boring.* The borings of *L. plumula* differ in important respects from those of *Botula*; there are also differences between the borings of various species of *Lithophaga*. As shown in fig. 13, the inner half of the boring is a smooth cavity within the rock, but the outer half has a calcareous lining laid down by the animal. Beginning as an extremely thin layer, this increases in thickness towards the opening, which, in *L. plumula* though not in

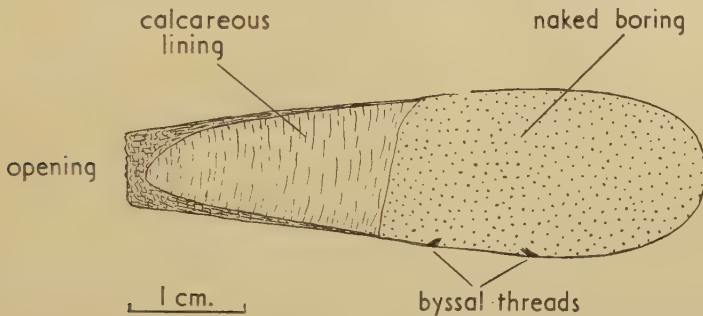


FIG. 13. *L. plumula*, boring opened lengthways (except for opening), showing extent of calcareous lining and positions where byssal threads attached.

all species, is round in section. The bounding layer varies greatly in thickness, from 0.5 to over 3.0 mm. in borings all some 8 cm. long. This doubtless depends on the age of the animal; after it attains maturity growth will slow down, but secretion of this calcareous layer, which must be carried out by the protruded inner lobes of the mantle forming the siphonal extension, continues. Judging by the thickness of the calcareous layer, its secretion also varies according to the nature of the rock surface and the presence of adjacent openings. Typically the layer is the same thickness all around the opening, but where there is a cavity in the rock greater secretion occurs on that side. Especially near to the opening, this secreted lining often contains cavities with contained organic matter. Apparently foreign particles, including organic debris and mucus, are incorporated in the calcareous matrix.

(f) *Method of boring.* *Lithophaga* is attached within the boring by byssus threads. The position of these is shown in fig. 13. They consist, as in *Botula*, of anterior and posterior groups, but each consists of only a few threads attached exclusively to the 'mid-ventral' region of the boring (which varies in position because the animal rotates) and not extending widely on either side of this as they do in *Botula* (fig. 5). Moreover, all the threads emerge from between the shell valves at the same place, as already noted and figured

by Kühnelt (1930) in *L. lithophaga* and by Otter (1937) in *L. cumingiana*. In a specimen of *L. plumula* of shell length 5.1 cm. and inhabiting a boring 6.3 cm. long, the anterior group of threads was attached 1.9 cm. from the head of the boring and the posterior group 1.1 cm. farther back. The former consisted of 12 and the latter of 6 threads. The disposition of the pedal and byssal muscles is essentially similar to that in *Botula* (fig. 6, A and B).

It has been shown that *Botula* bores straight into rock without rotating; this appears true of certain species of *Lithophaga*, e.g. *L. cumingiana*, where the boring is not circular in section (Otter, 1937). But *L. plumula* certainly rotates in the boring. Evidence is provided by the circular outline of the boring when seen in section (fig. 14, C), by the even distribution of the secreted calcareous lining in the 'posterior' half (fig. 13) (in *L. cumingiana* (fig. 14, B) this is restricted

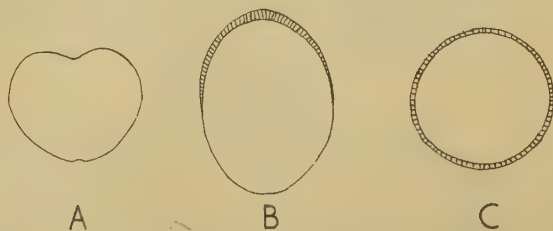


FIG. 14. Cross-sections of 'posterior' regions of borings of A, *Botula falcata* (animal does not rotate and no calcareous lining); B, *Lithophaga cumingiana* (animal does not rotate, calcareous lining 'dorsally'); C, *L. plumula* (animal does rotate in boring so this circular and calcareous lining not confined to one side). Varying magnifications. B, after Otter (1937).

to the 'dorsal' side (Otter, 1937)), and by the frequent presence of remains of byssal attachments all around the inner surface of the boring. Conclusive proof was, however, furnished in one instance where a boring was opened to expose the hinge line of the contained animal. During the succeeding days this animal was seen to rotate in the boring until only the side of one valve was visible; then it very slowly moved back until the mid-dorsal region was again exposed. This had represented a turning movement through about 90° and back again. Such movements would present no difficulty because of the smooth surface of the wall of the boring and of the fact that very few byssus threads would have to be detached and replaced to enable the animal to rotate without losing the necessary hold on the boring.

Compared with *Botula*, *L. plumula* attaches itself by similar groups of byssus threads, but these are less numerous and more concentrated in area of attachment. They will suffice for pulling the shell forward against the head of the boring but certainly not for grinding into the rock. Application of the protruded tissues of the fused inner lobes of the mantle edge, together with production locally of some acid secretion, are primarily responsible for penetration into the rock. Evidence against the occurrence of mechanical boring, such as occurs in *Botula*, may thus be summarized: (a) lack of deep abrasion of shell; (b) great reduction and localization of byssus threads. Evidence in favour of chemical boring is similarly summarized: (a) exclusively calcareous

nature of rock bored by all species of *Lithophaga*, (b) protrusion anteriorly of fused inner lobes of mantle edge, (c) presence in these of areas of glandular tissue. Of these points only the last requires further elaboration.

(g) *Glandular areas*. Carazzi (1892, 1903), List (1902), and Pelseneer (1911) all drew attention to the presence of glandular areas within the mantle of species of *Lithophaga* which they considered were, or could be, responsible for production of an acid secretion, this acting, according to Kühnelt (1930), by direct application of the mantle margin to the calcareous rock. Similar glands have been found in *L. plumula*. As in all species where they have been described, they consist of anterior and posterior areas situated in the regions where the mantle lobes separate, i.e. anterior and posterior to the mantle isthmus (as defined by Owen, Trueman, and Yonge, 1953). Pelseneer (1911) also describes a second pallial gland in *L. gracilis* which he says is absent in other species, including the Mediterranean *L. lithophaga*. This is a median, unpaired gland situated immediately anterior to the mouth and so opening into the mantle cavity. No such gland occurs in *L. plumula* (or in either species of *Botula*). Judging from Pelseneer's figure (there is some confusion in the lettering of the two sets of glands in his plates), it appears very similar to the pallial gland found in the same position in species of *Pinna* (see Yonge (1953b) for full discussion). Here also it does not occur in all species of the genus, being present, for instance, in *P. nobilis* and *P. carnea* but absent or poorly developed in others. It is most probably excretory; its absence appears associated with the presence of well-developed pericardial glands and vice versa (White, 1942). This may well be so in *Lithophaga*; the gland can hardly assist in chemical boring as suggested by Pelseneer, because it opens into the mantle cavity and any secretion must be ejected together with pseudofaeces from the inhalant chamber or else pass through the ctenidia and so leave with the exhalant current.

The anterior and posterior glandular areas consist, as described and figured by Carazzi, List, and Pelseneer, of folds of glandular epithelium. They open on to the surface of the fused inner lobes of the mantle edge in the depths of the anterior and posterior embayments, i.e. *outside* the mantle cavity. Their probable origin will be considered before proceeding to discuss possible present function.

Examination of *Modiolus modiolus*, obtained living from the Millport marine laboratory, has been most helpful when considering the origin of these glands. The possible evolutionary processes responsible for the appearance of the heteromyarian Mytilacea have been outlined elsewhere (Yonge, 1953a) and will be further discussed later in this paper. It is sufficient here to note that, apart from the more specialized borers, the end-result is the appearance of bivalves living attached, usually to hard substrata, by means of byssus threads, so that the animal is vertically disposed (i.e. unlike the horizontally attached *Ostrea*, *Anomia*, &c.). There is some flattening from above to below of the mantle/shell and to a less extent of the body, also considerable posterior extension, primarily of the mantle/shell, which is associated with the hetero-

myarian condition (Yonge, 1953a). When the valves separate, a long stretch of mantle margin is exposed along the topographically upper surface (morphologically posterior because behind the mantle isthmus and ligament). The exposed tissues consist of fused inner lobes of the mantle edge fringed by the middle lobes. These extend from the posterior margin of the exhalant aperture to the depths of the embayment where the middle mantle lobe, together with the periostracal groove between this and the outer lobe, bend around from one valve to the other.

In *Modiolus* (see fig. 6, c) this 'dorsal' exposure of the mantle margins

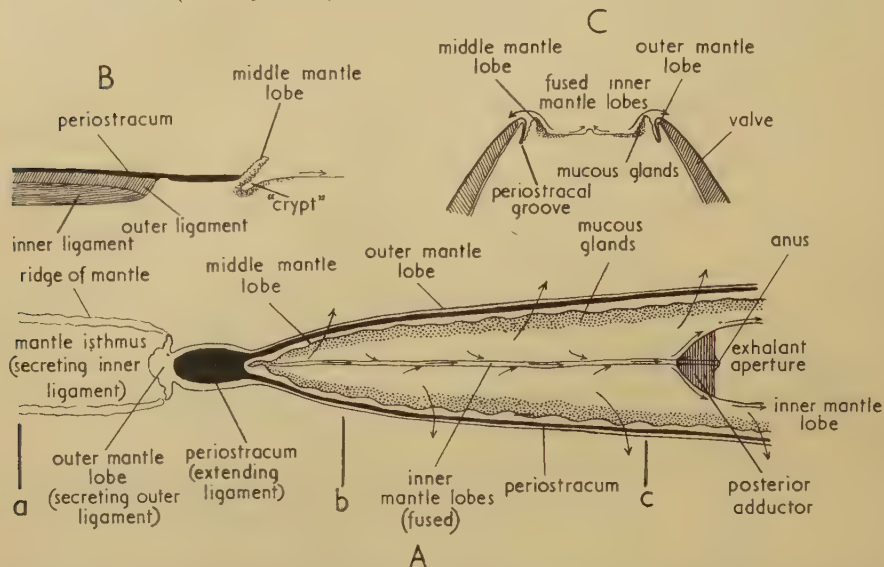


FIG. 15. *Modiolus modiolus*, semi-diagrammatic figures showing mid-dorsal tissues between ligament and exhalant aperture. A, dorsal view (shell removed); B, longitudinal section between lines *a* and *b* in A, showing layers of ligament; C, transverse section (shell present) along line *c* in A, showing outer, middle, and fused inner lobes of mantle edge with periostracal groove between the two first. Arrows indicate direction of ciliary currents removing sediment, stippling presence of mucous glands.

extends for over half the length of the animal, for some 6 cm. in the case of an animal with a shell 10.5 cm. long. Along the line of their fusion, the inner mantle lobes are raised into a median ridge, as shown in fig. 15. When the valves separate, sediment will fall on to these exposed tissues or material be carried on to them by water movements. This must be removed and, as usual in the lamellibranchs, mucus and cilia combine to effect this. As shown stippled in fig. 15, mucous glands are particularly numerous on the inner surface of the middle lobe of the mantle edge. Arrows indicate the direction of ciliary currents. On either side of the median ridge formed by fusion of the inner mantle lobes cilia beat towards this and particles are carried posteriorly along the ridge and then on either side of the exhalant aperture for final removal in the exhalant current. But over the greater part of the surface cilia beat outwards so that particles, embedded in mucus as they pass over the

middle mantle lobes, are carried on to the surface of the valves, where, as first pointed out to the author by Mr. G. Owen, accumulations are formed which, although less consolidated, are essentially similar to those on the shells of *Botula* and *Lithophaga*.

The abundant mucus required is produced in sub-epithelial glands which communicate with the surface by conspicuous ducts, often swollen with secretion, which run between the cells of the ciliated epithelium. In sections stained with haemalum and Alcian blue the glands and ducts colour reddish-purple with the former, but the extruded mucus which usually remains after fixation as a layer on the surface of the epithelium stains blue. The gland cells are of the granular acidophil type which List (1902) distinguished from others which are basophil and with hyaline contents.

Where the middle lobe of the mantle edge bends round in the depth of the embayment posterior to the ligament, the inner surface extends forward to form an irregular pouch or 'crypt' (shown in fig. 15, B). The roof of this pouch represents anterior extension of the inner wall of the middle mantle lobes, the floor the forward extension of the fused inner mantle lobes. All secretion produced by the accumulation of mucous glands is carried by ciliary currents posteriorly and then laterally.

The posterior glandular area described by previous workers in species of *Lithophaga*, although larger, occupies precisely the same position. A similar area is present in *Botula*. In addition there are anterior glandular areas of precisely the same type in both of these genera. Owing to changes in shell form, which are discussed later, in both of these there is some dorsal exposure of the mantle edges anterior as well as posterior to the mantle isthmus and so some original need for removal of sediment from this region also. In *Botula* the anterior glandular area is small, in *Lithophaga* it is very large, and it is this gland which is probably responsible for at least the initial softening of the calcareous rock.

As already described and shown in figs. 11 and 12, the fused and much thickened inner lobes of the mantle edge are here protruded. The mucus secreted by the anterior glandular area will thus be applied to the surface of the calcareous rock. Although no free acid could be detected, thus confirming the findings of Kühnelt, the presence of an acid mucus must be postulated. Kühnelt kept specimens of *L. lithophaga* in glass tubes, where they extended their siphons while anteriorly the thickened mantle margins were protruded and usually pressed against the wall of the tube—as here observed *in situ* with *L. plumula*. Kühnelt further experimented by placing a piece of cuttlefish bone in a tube in front of an animal. Over a period of 14 days the protruded mantle edges touched the cuttlefish bone, on which an impression was left which could not have been produced mechanically and must have been due to slow but continuous chemical action.

It is probable that in *Lithophaga* the posterior glandular areas have a similar function, serving to widen the boring which the anterior glands deepen. Movements of the valves will remove partly dissolved limestone and keep the walls

of the boring smooth. To that extent, but no more, there will be some mechanical action; Kühnelt reports occasional erosion of the periostracum round the umbones in *L. lithophaga*, and this is true of *L. plumula*. But the byssal attachments, in such contrast to those of *Botula*, are too few and too weak to permit significant grinding action against the rock.

The general direction of the ciliary currents on the extruded anterior tissues is ventral, as shown in fig. 11, and then posterior. But the movements of the animal in the confines of the boring, including opening and closing of the shell, must cause mucus with dissolved or finely granular limestone to pass laterally on to the surface of the valves. This matter is further discussed later.

In origin, therefore, the glandular areas may be regarded as enlargements of the mucus-secreting strip associated with the inner side of the middle lobe of the mantle edge. The increased production of mucus served to entangle the sediment and other particles which fell on to the dorsally exposed pallial tissues, posterior only in *Modiolus* but anterior and posterior in *Botula* and *Lithophaga*. In the last genus—or subgenus—secretion of an acid mucus and extrusion of the anterior as well as of the posterior pallial tissues permitted chemical penetration of calcareous rock with the minimum of mechanical aid. But the shape of the shell was such that, with adequate byssal attachment, boring could be purely mechanical, as it is in *Botula*. In this genus the mucus secreted both anteriorly and posteriorly will entangle the particles of rock ground away by the forward and lateral pressure of the valves. But only material accumulated posteriorly will become attached to the shell valves; the other will pass into the mantle cavity and be expelled with the pseudofaeces.

OBSERVATIONS ON OTHER SPECIES OF *LITHOPHAGA*

It is appropriate to refer now to other species of *Lithophaga* which have been examined. In 1951 observations were made on *L. nigra* at Bermuda, where it is common in the aeolian limestone. The shell is very like that of *L. lithophaga*, i.e. dark brown with very little, or no, material adhering to it. The borings are flask-shaped with an elongated, somewhat dumb-bell shaped opening, similar to that of *L. lithophaga* (Kühnelt, 1930) and also of *L. cumingiana* (Otter, 1937). The borings of both the first two species differ from those of the last species, and those of *L. plumula*, in the absence of any secreted calcareous lining round the opening and throughout the posterior half of the boring. Attachment by byssus threads, never numerous, is confined to the 'mid-ventral' line of the boring, and this fact, together with the shape of the opening, indicates that *L. nigra* does *not* rotate during boring; it must work its way straight in, as does *Botula* (Kühnelt disagrees with Carazzi in thinking that *L. lithophaga* rotates in the boring; but it is difficult to accept this view). However, although in cross-section the boring is somewhat 'higher' than it is 'broad', there are, unlike *Botula*, no 'dorsal' and 'ventral' ridges. But the shell of *L. nigra* is almost circular in cross-section, while the method of boring, by local application of acid instead of mechanical attrition, is unlikely to leave ridges.

The absence of these ridges, the weakness of the byssal attachments, and the calcareous nature of the rock bored, all provide evidence that this species bores chemically, but it is apparently confined to softer rocks than *L. plumula*. Although borings were carefully opened to expose the contained animal, in the same manner as were those of *L. plumula*, only rarely were the siphons seen expanded. The valves did then separate anteriorly but the mantle edges were never seen protruded.

An interesting collection of specimens of *L. aristata* was received through the kindness of Mr. Martin Routh, who had collected them from the coast of tropical West Africa in 1951. The rock bored, specimens of which he also sent, was a beach rock or calcareous 'sandstone' very like the aeolian limestone from

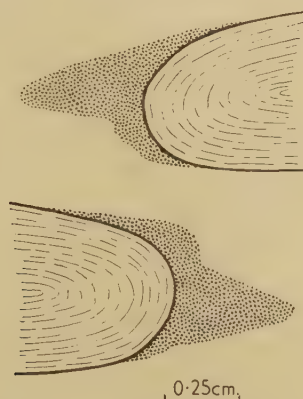


FIG. 16. *Lithophaga aristata*, posterior ends of valves, left one above and right one below, viewed from inside to show asymmetrical spur-like calcareous projections (stippled).

Bermuda. In this species the shell is covered with amorphous calcareous deposits, while posteriorly each valve is prolonged by a spur-like calcareous plate (fig. 16). These are disposed one above the other so that the valves continue to make perfect closure. There is a well-developed calcareous lining around the opening and the posterior half of the boring. The opening is dumb-bell shaped and very elongated dorso-ventrally. Here also all available evidence indicates that the animal does not rotate in the boring.

MATERIAL ADHERENT TO THE SHELL

Kühnelt (1930) pointed out that the calcareous incrustations which are so characteristic of the shells of *Lithophaga* s.s. are of two types, which he distinguished as amorphous and crystalline. The former he described as present in the Mediterranean *L. lithophaga* but as being much thicker in the West Indian *L. appendiculatus*. When the calcium carbonate was removed with acid the residue consisted of a brown mass, not of conchyolin, with contained fragments of algae, diatoms, and other organic remains. Kühnelt thought these incrustations were not secreted as such by the bivalve but were cemented

together by a secretion. The crystalline incrustations contained no such organic matrix; they occurred on the inner walls of the boring and also on the hind end of the valves in some species. He noted further that while both types of incrustations can occur in the same species, the amorphous type never occurs under the crystalline type, while the opposite condition is common.

In his description of rock-destroying organisms in relation to coral reefs examined in the course of the Great Barrier Reef Expedition, Otter (1937), without knowledge of the work of Kühnelt, distinguished between three areas of calcareous deposition on the shell: (a) postero-dorsally, (b) antero-ventrally, and (c) dorsally. He noted that in *L. hanleyana* accumulations on the first of these areas are well developed and striated; he described them, indeed, as forming 'a close-fitting operculum to the burrow'. The condition he describes is very similar to that in *L. plumula* and illustrated in figs. 7 and 10. These constitute one example of the crystalline deposits described by Kühnelt, the asymmetrical spur-like plates in *L. aristata* being another. Both are distinct from the amorphous deposits found in other areas and exclusively in many species such as *L. lithophaga* and *L. nigra*, together with *L. cumingiana*, *L. obesa*, and *L. teres*, described by Otter.

Interpretation of the origin of these deposits is aided by examination of *Botula*. Here there are no calcareous deposits, but, as already described and shown in figs. 1-3, accumulations of material consisting of fragments of byssus threads, algae, sand grains, &c., cover the posterior regions of the valves. In *Botula* there is some extrusion of the mantle margins posterior to the ligament but none anteriorly. The deposits on the shell consist of foreign matter agglutinated in mucus from the posterior glandular area. There is an anterior glandular area, but secretion from this is carried ventrally and then posteriorly, and never gets carried over the surface of the valves.

In *Lithophaga*, where mantle tissues are protruded both anterior and posterior to the ligament, there are two areas of deposition on the valves. This is particularly well shown in species which do not rotate within the boring, e.g. *L. cumingiana*, where Otter's figure of a specimen in the burrow (1937, text-figure 2) shows two distinct areas, extending postero-ventrally one from the anterior and the other from the posterior glandular area. There seems no question as to the origin of these amorphous deposits, namely, mucus from the glandular areas in which particles, primarily calcareous debris from the wall of the boring or precipitated after initial solution by acid, accumulate. In *Botula* the posterior accumulations have similar origin but are, of course, non-calcareous.

The crystalline deposits are formed by the walls of the siphons. Secretion of calcareous matter by these may extend the shell valves adventitiously in the manner shown in fig. 10, where the calcareous plates so formed can be seen projecting posteriorly. Only the walls of the siphons, i.e. the locally enlarged and extended inner lobes of the mantle edge, could produce these structures; the outer lobe of the mantle edge which secretes the shell is covered by thick periostracum, and these calcareous plates are formed on the surface of the

periostracum. As the valves enlarge, so will these extend *under* the calcareous plates, which are themselves being added to by the action of the siphonal walls.

These adventitious additions to the shell, which occur in a number of species of *Lithophaga*, take two major forms, the one plume-like as in *L. plumula*, the other asymmetrical and spur-like as in *L. aristata*. Where they are present a calcareous lining is also laid down around the opening and within the posterior half of the boring. This may also be formed in species where there are no crystalline deposits on the shell, e.g. in *L. cumingiana*. In other species, such as *L. lithophaga*, *L. nigra*, and *L. teres*, there is no secretion, either on the boring or on the shell, by the siphons.

The capacity of the siphons to secrete calcareous salts is not uncommon. It occurs habitually in *Rocellaria* (*Gastrochaena*), as described by Sluiter (1890) and Otter (1937). In *Teredo* the projecting siphonal tubes, which are separate, are usually naked, but under certain circumstances they can lay down calcareous tubes around themselves, as shown in the case of *T. norvegica* by Yonge (1927).

There are clearly considerable differences between the various species of *Lithophaga* s.s., all of which bore into calcareous rock. Otter (1937) arranged the species he examined into the following series: *Modiolus cinnamomeus* and *Lithophaga argentea*; *L. teres*; *L. cumingiana* and *L. obesa*; *L. hanleyana*; presenting, he considered, increasing degrees of specialization. The first two species inhabit shallow burrows and have no calcareous deposits on the shell or the boring, the last named bores deeply and has crystalline deposits on both shell and boring. But the position is not so simple as he thought; both *L. nigra* and *L. aristata*, the former with the minimum of deposition on the shell, the latter with both amorphous and spur-like crystalline deposits on the shell and a lining to the boring, bore into similar, very friable limestone. The latter does not seem the more specialized. There is no obvious correlation between secretion by the siphonal walls and the type of rock bored.

Thiele (1935), on the basis of the crystalline deposits on the shell, has subdivided the genus *Lithophaga* s.s. into three sections: (1) *Lithophaga* s.s. where there are no such deposits, e.g. *L. (L.) lithophaga* (Linné); (2) *Myoforceps* P. Fischer 1886 with the smooth projecting calcareous deposits found in *L. aristata*; (3) *Diberus* Dall 1898 with plume-like incrustations as in *L. plumula*. It is still questionable whether the form of the deposits is a sound basis for classification, depending as it does on the secretory activity of the siphons which may be influenced environmentally, as it certainly is in *T. norvegica*. Much work, both in the laboratory and still more in the field, remains to be done on this important genus with species so widely distributed throughout warmer seas.

EVOLUTION OF THE BORING HABIT IN THE MYTILIDAE

In the Mollusca, whereas the body is (at least primitively) bilaterally symmetrical, the mantle/shell has a radial symmetry, increasing peripherally (Yonge, 1952, 1953, *a* and *b*). Thus when discussing form in the Lamelli-

branchia, the two must be considered separately. For instance, the form of the shell in different species can be related by the use of *radial* co-ordinates but the form of the bodies only by *rectangular* co-ordinates (Yonge, 1952). The final form assumed by the animal is the result of interaction of the growth of the body on the one hand and of the mantle/shell on the other. The most convenient way to consider problems of form is by reference to axes, or projections from the curved surface of the mantle/shell, in the sagittal plane, namely,

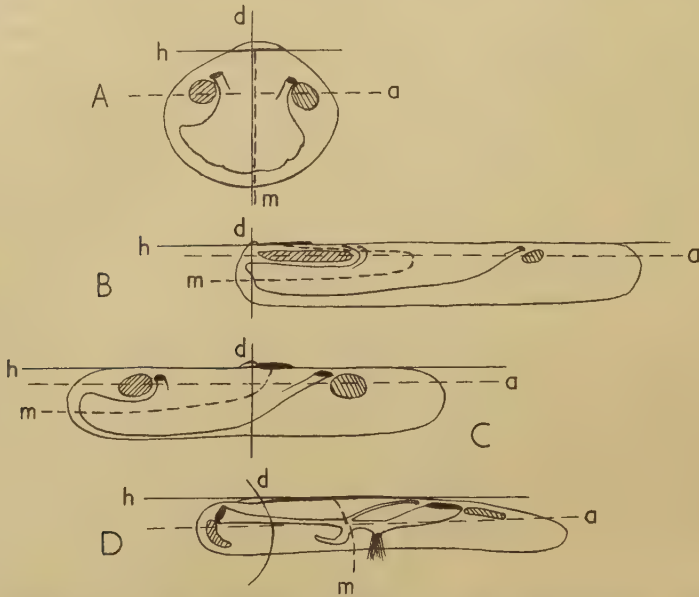


FIG. 17. Elongation in Lamellibranchia. Diagrams showing different ways in which length can be attained. A, *Glycymeris* (dimyarian with equilateral shell); B, *Ensis* (dimyarian with mantle/shell extended posteriorly, secondary effects on body); C, *Tagelus* (dimyarian with mantle/shell extended both anteriorly and posteriorly, secondary effects on body); D, *Botula* (dimyarian after being heteromyarian, lengthening due to this followed by effect of tangential component in growth of mantle/shell). For full description see text. *a*, *m*, antero-posterior and median axes of body (broken lines); *h*, *d*, hinge line and demarcation line of mantle/shell (unbroken lines).

the antero-posterior axis and the median axis of the body together with the hinge line and the demarcation line of the mantle/shell. 'Demarcation line' is used instead of 'normal axis' previously employed (Owen, 1953, *a* and *b*; Yonge, 1953, *a* and *b*), which is dubiously applicable to a projection from a curved surface. Starting from the umboes it represents the projection on to the sagittal plane of the line of maximum inflation of each valve, i.e. the region where the ratio of transverse to radial component in the growth of the mantle/shell is greatest (Owen, 1953*a*). Projections from the two valves coincide if the shell is equivalve. This line divides each lobe and valve of the mantle/shell into anterior and posterior territories which are approximately equal only in equilateral shells. (It is a pleasure to acknowledge the help of

Dr. L. R. Cox, F.R.S., and of Mr. G. Owen in clarification of these problems.) Hinge line rather than hinge axis is used to conform with demarcation line.

Starting with an equivalence and equilateral dimyarian such as *Glycymeris* (fig. 17, A), the antero-posterior axis of the body, which runs from mouth to anus, and the hinge line of the mantle/shell are parallel, while at right angles to them are the median axis of the body and the demarcation line of the mantle/shell which, in this case, effectively coincide. The median axis may be

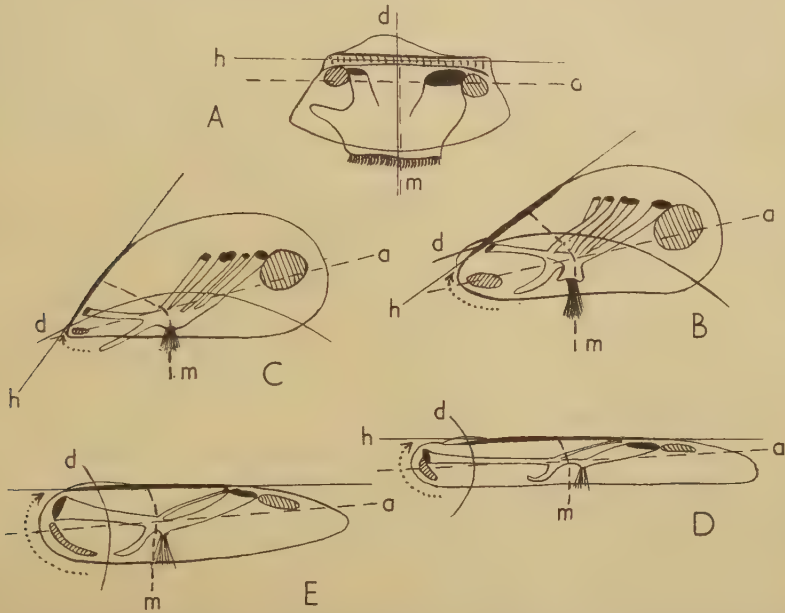


FIG. 18. Evolution within the Mytilidae. Diagrams showing adductors and foot with byssus and retractors, together with antero-posterior and median axes of body and hinge and demarcation lines of mantle/shell. A, *Arca*, byssally attached dimyarian (not Mytilidae); B, *Modiolus*; C, *Mytilus*; D, *Botula*; E, *Lithophaga*. Effect of tangential component in growth of mantle/shell indicated by dotted arrows.

defined as a line running from the mid-dorsal point of the body, i.e. the centre of the mantle isthmus, through the middle of the base of the foot to the mid-point ventrally of the foot or the byssus (Yonge, 1952, 1953a, 1953b). Thus in *Glycymeris* (fig. 17, A) it divides the headless body into almost equal anterior and posterior halves. The demarcation line, defined above, similarly divides each mantle lobe and valve into anterior and posterior territories.

As already pointed out (Yonge, 1953a), the symmetry both of body and of mantle/shell may be altered (1) by changes in the form of the mantle/shell affecting that of the body, or (2) by changes in the body influencing the mantle/shell. The former is well displayed in the Solenidae (Yonge, 1952, 1953a) where, as shown in fig. 17, B, the pulling out posteriorly of the mantle/shell by greater growth in that region has major effects on the form of the body. Where, however, the body makes permanent attachment to the substratum by a byssus, as it does for instance in *Modiolus* (fig. 18, B), then

changes in form affect first the body and only secondarily the mantle/shell. Under these conditions the ventral surface of the foot (or the byssus) must be regarded as *the fixed point*; whatever else may be affected, this mid-ventral region of the body remains constant in relation to the environment.

Attachment of this character may have little effect on the proportions of the body, e.g. in *Arca* (fig. 18, A). More usually, however, and for reasons discussed elsewhere (Yonge, 1953a), it brings about reduction of the anterior end of the body, with secondary reduction of the mantle/shell, including the anterior adductor. With the exception of *Acostaea* (*Mulleria*), where the anterior adductor is lost following cementation by one or other valve, all monomyarians are, or, it is contended, were originally, byssally attached (Yonge, 1953a), and passed through a heteromyarian condition probably not unlike that now seen in *Modiolus*. In this genus the anterior half of the body is reduced (note position of the median axis in fig. 18, B), the foot and byssus ventrally and the ligament dorsally appearing to have moved anteriorly. Hence the median axis is curved while the antero-posterior axis is tilted downward anteriorly (cf. figs. 18, A and B).

Passing to the mantle/shell, the anterior adductor is much reduced and the posterior adductor correspondingly enlarged. The umbo is near the anterior end and the hinge line is no longer parallel to the long axis of the shell but is tilted downward anteriorly to an even greater extent than is the antero-posterior axis of the body. The hinge line is straight and unusually long, for reasons which are discussed below. Finally, the demarcation line, at right angles to the hinge line in *Glycymeris*—and also in the Solenidae (fig. 17, B)—here cuts this at an acute angle and then curls round in an anterior direction. This curvature is due to the effect of a tangential component in the growth of the mantle/shell which is so conspicuous in *Glossus* (*Isocardia*), as fully described and analysed by Owen (1953a, 1953b).

The tangential component in the Mytilidae, although not so immediately obvious, is no less significant than in *Glossus* and, since it is an all-important factor in the evolution of *Botula* and *Lithophaga*, must be discussed. The effects on the ligament may first be mentioned. As in *Glossus* (and also in *Chama*) the swinging round anteriorly of the mantle/shell (indicated by the arrows in figs. 6 and 18) causes a splitting of the anterior end of the ligament. (Examination from within of the anterior end of the ligament shows this very clearly.) This splitting was noted by Trueman (1950) in *Mytilus edulis*, but he regarded it as due to 'slight dorsal arching coupled with the increased gape of the valves'. But it is without doubt a consequence of the tangential component in the growth of the mantle/shell as stated by Owen (1953a). The ligament is further affected by being extended posteriorly. Where the hinge line is curved, as in *Glossus* and *Chama*, the effect of posterior extension is offset by anterior splitting. But in the Mytilidae the hinge line is unusually straight. This may or may not have been due initially to the tangential component in growth, but it must have been greatly accentuated by this. It is certainly not merely due to flattening of the dorsal surface of the shell, or the ligaments of

bivalves, such as *Ensis* or *Tagelus* (see fig. 17, B and C), would be even longer. In the crowded environment occupied by many of the Mytilidae, notably species of *Mytilus*, the capacity to push the valves apart with unusual force, due to possession of a long ligament, may well be of great value.

But the tangential component has had more far-reaching effects. Progressive reduction of the anterior half of the animal leads to the type of monomyarian exemplified by such bysally attached Anisomyaria as *Pedalion*, *Pinctada*, and species of *Chlamys*. The animal lies on its right valve while the right anterior pedal retractor atrophies so that the foot, retained solely for planting the byssus, can migrate anteriorly without occluding the mouth (Anthony, 1905; Yonge, 1953a). This would happen did both retractors persist because they diverge from the foot towards the shell valves with the mouth-opening

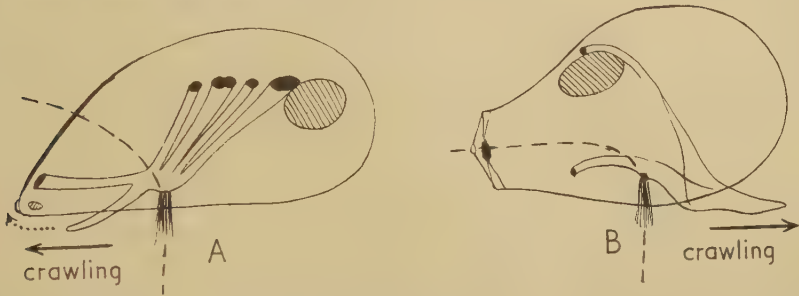


FIG. 19. A, *Mytilus*; B, *Lima*; diagrams showing how effect of tangential component in growth of mantle shell in *Mytilus* permits crawling hinge foremost despite reduction in anterior half of body (left of median axis denoted by broken line). In *Lima*, where no tangential component, foot is reversed or mouth would be occluded; hence crawls with hinge hindmost.

lying between them. The one group of monomyarians which retain bilateral symmetry with the foot as an organ of locomotion is the Limidae, where the valves remain vertically disposed. And in this family the *foot is reversed* (Seydel, 1909; Studnitz, 1930; Yonge, 1953a), the animals crawling with the *hinge hindmost*. There is no tangential component of mantle/shell growth in the Limidae where the ligament is very short. The presence of this component appears to have been of prime importance in the Mytilidae by permitting the maximum degree of heteromyarianism—found in *Mytilus*—without reduction of the foot or loss of the power of crawling with the hinge foremost. The swinging round anteriorly of the tissues has enabled the anterior pedal retractors to persist without occluding the mouth-opening. (In *Dreissena* this problem has been overcome by the formation at the anterior end of the shell of shelves to which the anterior adductor is attached.) Comparison is made in fig. 19 of the essential differences between *Lima* and *Mytilus*.

Within the Mytilidae, as shown in fig. 18, the extreme heteromyarian condition found in *Mytilus* represents the culmination of evolutionary change in one direction, while conditions in *Botula* and *Lithophaga* do so in another. Their effects upon the mantle/shell of reduction of the anterior half of the body are in effect completely offset by increase in the tangential component

in growth which, as shown by the dotted arrows in fig. 18, has had a far greater effect than in *Modiolus*. The forward tilting of the hinge axis is counteracted and an elongate shell with parallel dorsal and ventral margins is produced. Anteriorly the mantle margins are carried on to the dorsal surface, with the consequent appearance there of glandular areas and so, eventually, of the means of chemical boring in *Lithophaga*. The anterior adductor increases in size and the posterior adductor diminishes, at least relatively; so from a somewhat triangular heteromyarian an elongate dimyarian is formed.

Superficially this elongate shell resembles that of a solenid such as *Ensis* or an extended tellinid such as *Tagelus* (Yonge, 1949, 1952). The different ways in which these three apparently very similar types of shell have been evolved are indicated in fig. 17. The elongate form of *Ensis* (B) has come about owing to displacement posteriorly of the centre of maximum shell growth (an intermediate stage between *Glycymeris* and *Ensis* is provided by *Siliqua*, where the maximum shell growth is postero-ventral (Yonge, 1952)). *Tagelus* (C) represents a simple pulling out of each end due to the presence of two growth centres anterior and posterior to the median vertical axis. In other respects it is a typical member of the Tellinacea (Yonge, 1949). In both *Ensis* and *Tagelus* it will be noted that although the animal is elongated, the hinge line and the demarcation line of the mantle/shell have the same relations to one another as they do in *Glycymeris*. It is the median axis of the body which has been affected, the foot being now protruded anteriorly. In the case of *Ensis* the median axis had been bent into a U shape because the middle of the body has been pulled posteriorly. In *Tagelus* there is no such effect, but, as in *Ensis*, elongation of the mantle/shell has brought about anterior prolongation of the foot.

Both in *Ensis* and in *Tagelus* the anterior and posterior halves of the body, though pulled out in different ways, remain of approximately the same size. To a large extent this is also true of *Botula* and *Lithophaga* (D) but for other reasons. The effect of tangential growth of the mantle/shell has been, first, to offset the effect of reduction of the anterior half of the body on the form of the mantle/shell; it has, second, been to allow the anterior half of the body to regain its original dimensions. This is best realized by reference to fig. 6, where comparison is made with conditions in *Modiolus*. It will be noted that the present form of *Botula* and *Lithophaga*, showing as it does clear evidence of tangential growth of the mantle/shell, could only have evolved by way of a heteromyarian condition; failing this the shell would have twisted round like that of *Glossus* or *Chama*. As it is, an elongate shell, superficially like those of *Ensis* and *Tagelus*, has been produced, but the ventral surface of the body, i.e. the foot and byssus, has remained a fixed point; the median axis of the body has not been bent forward ventrally as in those genera (cf. figs. 17, B, C, and D).

Having shown how elongate mytilids may be evolved, it remains for consideration how they assumed their present boring habits. It was certainly not—as is too often assumed—the boring habit which produced the form; it was

the elongate form which made boring possible. Like other boring Lamelli-branchia which are, or may be, byssally attached, such as species of *Tridacna* (Yonge, 1936) and of *Hiatella* (Hunter, 1949), the habit of boring was probably preceded by that of 'nestling'. The animals attached themselves in crevices which they may have widened or deepened by movements of the valves. This habit is found in various species of *Modiolus*, such as *M. cinnamomeus* described by Otter (1937). But apart from the Tridacnidae, where profound changes in the relationship of body and mantle/shell (Yonge, 1936, 1953a, 1953c) enable the animals to bore hinge foremost, it is only where the shell is long that true boring is possible. The animal then enters the rock anterior end foremost. In *Hiatella* the byssus is then lost (Hunter, 1949), but in *Botula* it is retained and, so attached, the animal is able to force its way into the rock. In *Lithophaga* use has been made of the glandular areas, evolved originally in the manner already recorded, to produce an acid mucus, so that calcareous rocks are entered chemically. With this change of the mode of boring (because initially all boring in the Mytilidae must surely have been mechanical) the extent of byssal attachment has been reduced.

The force needed for boring is provided by the posterior byssal retractors and the ligament. The former deepen the boring by forcing the anterior end of the shell against the rock, the latter widen it by forcing apart the shell valves. In this the Mytilidae differ profoundly from the pholads. There, attachment being provided by the sucker-like foot, boring proceeds by rocking of the valves about the median dorso-ventral axis, due to alternate contraction of the two adductors, the shell gaping at both ends. The ligament is either lost or only a functionless vestige is retained (Purchon, 1954), the valves being in effect separate and only in contact dorsally, and in some cases (e.g. *Teredo*) also ventrally, by way of rounded articulating surfaces.

The exceptionally long ligament of the Mytilidae, with consequent powerful opening thrust, represents another preconditioning factor which made boring—as executed by *Botula* and *Lithophaga*—possible. What enables *Mytilus* or *Modiolus* to open the shell valves against the pressure of other individuals provides the force which widens the boring in *Botula*, although now greatly assisted by the secretion of acid mucus in the case of *Lithophaga*. Elongation of the shell in these species has been accompanied by greater extension of the ligament, as shown in fig. 6. The length of the true ligament in relation to that of the shell is 40% in *Modiolus*, 50% in *Lithophaga* and *Botula*. But the true ligament (inner and outer ligament layers with superficial periostracum formed as described by Owen, Trueman, and Yonge (1953) and Yonge (1953b)) is extended posteriorly by a length of thick periostracum without underlying layers (the 'posterior cover' of Trueman (1950)). This is due to the elongation and lateral compression of the periostracal 'groove' in the depth of the embayment between the valves, as shown in fig. 15. Allowing for this addition, ratios of ligament to shell length rise to 52% and 58% respectively.

Elongate shell form, including lengthened ligament, with mode of attach-

ment and general habit of life, all, therefore, represented preadaptations to mechanical boring in the Mytilidae. Extension dorsally of the mantle edges was accompanied by increased mucus secretion to aid in cleansing. Protrusion of these tissues and direct application by them of an acid mucus provided the means of chemical boring in *Lithophaga*. Change in form made possible change in habit.

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The Sheath of Spicules of *Leucosolenia complicata*

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With two plates (figs. 1 and 2)

SUMMARY

Dilute hydrochloric acid, carbonic acid, and potassium nitrate solutions dissolve the spicule calcite revealing a thin, partially contracted sheath. Corrosion by potash solution, however, produces a rigid, brittle 'sheath', which consists of the true sheath and an inorganic deposit laid down on its inner surface as the calcite dissolves away.

The oscular rays, gastral rays, and curved monaxons corrode much more rapidly than the basal rays and slender monaxons in potash solution, and the corrosion is most noticeable on the surfaces transverse to the optic axis, particularly when dilute potash is used. Potassium nitrate solution and water, however, corrode the surfaces parallel to the optic axis, whereas in hydrochloric and carbonic acid solutions the calcite dissolves uniformly all round the rays.

When spicules bearing calcite crystals are corroded, the calcite of the spicules dissolves more rapidly than the crystals, which then remain attached to the sheath and appear to have crystallized upon it, though attempts to crystallize calcite upon isolated sheaths, or sheaths supported by the inorganic deposit, have been unsuccessful. The evidence suggests that when crystals form on the spicule surface they are oriented by crystallizing on the calcite through perforations in the sheath.

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INTRODUCTION

IT has long been known that concentrated solutions of potash dissolve calcareous sponge spicules, leaving behind a distinct sheath outlining the original surface of the spicule. Bütschli (1901) believed that this sheath was largely inorganic in composition since dilute hydrochloric acid dissolved all but a sparse, presumably organic, residue, and he decided that the inorganic constituent was probably calcium carbonate, though later (1907) he learned from Hofmann that calcium hydroxide is precipitated when potash and

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calcium carbonate react together. The inorganic part was clearly an artifact for (a) it lay across the ends of broken rays, (b) it was insoluble in the very dilute hydrochloric acid which, apart from the organic residue, dissolved the untreated spicules completely, and (c) it appeared around fragments of mineral calcspar left in strong potash solution. However, Minchin and Reid (1908) misinterpreted Bütschli's paper and assumed that the true sheath of untreated spicules also contains the inorganic constituent, and the same error is found in Minchin's summary (1909). To clear up the confusion thus caused and to determine the regions of the spicule surface that are preferentially corroded, the present author has investigated the action of potash and other solutions on spicules of *Leucosolenia complicata* (Minchin, 1904), an account of which is given below.

The crystallization experiments described earlier (Jones, 1955) have also been continued in order to determine the possible role played in them by the true spicule sheath. It was shown that crystals develop on the surfaces of spicules left in a shallow solution of calcium bicarbonate, the form of the crystals being constant with the experimental conditions employed, but their siting being governed by the type of prior corrosion suffered by the spicules during the process of isolation from the sponge. Thus potassium nitrate solution corrodes the surfaces parallel to the optic axis (o.a.), whereas potash solution appears to attack preferentially the o.a.-transverse surfaces, and the subsequent initiation of crystal growth is thereby facilitated on one or other region. The influence of the true sheath on the crystal distribution was considered to be negligible because spicules much reduced in size by acid corrosion could nevertheless produce a crystal pattern like that of the normal spicule, even though their sheaths would be much distorted. The 'sheath' isolated by potash was also neglected because additional treatment with the potash resulted in an increase in the number of crystal sites, and not the decrease that would be expected were this 'sheath' hindering crystallization at any part of the spicule surface.

METHODS

Methods by which the spicules may be isolated and attached to the surface of a slide have already been outlined (Jones, 1955) and such slides of spicules were used in all the experiments described in this paper, the slides being dipped into the appropriate solutions in jars or petri dishes as required. The potash solutions were prepared immediately before use and changed at daily intervals whenever the experiment involved more than one day. The slides should not be left in contact with the 10% solution for longer than is necessary, because a white deposit, presumably a compound of the calcium leached out of the glass, forms on the surface after a few days.

For the crystallization experiments the same procedure was adopted as used previously (Jones, 1955). The glycerine jelly used as a mountant for spicules bearing crystals was prepared by G. T. Gurr Ltd., London.

RESULTS

Spicule corrosion by 5% potassium nitrate solution

As described in an earlier paper (Jones, 1955), potassium nitrate solution dissolves the spicule calcite mainly from the o.a.-parallel surfaces, splitting the rays into paired raylets. This poses a problem, for corrosion on the o.a.-parallel surfaces should merely make the ray thinner in the plane perpendicular to the optic axis, the o.a.-transverse surfaces diminishing in area as each successive layer on the former is removed. However, it is probable that the purity of the calcite varies throughout the spicule, and that the central core is impure and rapidly corrodes once it is exposed at the o.a.-parallel surfaces. Such a core of less pure calcite would agree with v. Ebner's (1887) interpretation of the spicule structure as well as with that of the protagonists of the organic axial filament (e.g. Minchin and Reid, 1908).

As with the corrosion by distilled water (see below), a thin sheath is left behind as the calcite dissolves. Its properties are described in the next section.

Corrosion by distilled water

In glass-distilled water (pH 6.5–7.0) the spicule corrodes slowly, the rays continuing to taper evenly towards their tips, though occasionally a slight fork is visible at the tip, together with corrosion pits on the o.a.-parallel surfaces. As the rays shorten a thin sheath, the true sheath already noted by several workers (see Minchin, 1909, for a summary), becomes evident and appears partially contracted. The sheath is flexible and waves about with movements of the ambient liquid, though it has some degree of rigidity for it can support the weight of a partially corroded spicule resting on the original ray tips. The spicules are very variable in their speed of corrosion, some being hardly reduced in size when others have almost completely dissolved. The variation is presumably attributable to differences in thickness of the sheath or in the purity of the calcite.

When the distilled water has been neutralized by the addition of about one-tenth of its volume of calcium bicarbonate solution prepared as usual (Jones, 1955), the division of the rays into paired raylets becomes much more evident. However, the corrosion in the potassium nitrate solution is faster than in water of the same pH (7.0) and temperature. For example, whereas $3\frac{1}{2}$ hours in the nitrate solution produced well-defined paired raylets at 17.5° C, neutralized distilled water formed none after $6\frac{1}{2}$ hours, though they were present the following morning, 17 hours later. This indicates that the potassium nitrate exerts a positive action in the corrosion.

No difference can be detected between spicules isolated by potash and by potassium nitrate solutions in respect of their corrosion by distilled water.

The action of potash solution

Bütschli (1901) used a concentration of 35% potash solution for his corrosion experiments, but this is unnecessary for even the $\frac{1}{2}$ % solution effectively

corrodes the spicules with the formation of a distinct 'sheath', though very slowly. Furthermore, the formation of crystals of double salts, which occupied Bütschli's attention for a considerable period (1907), is avoided if the solution be 10% or less in strength. In such a solution (1-10%) the calcite of *L. complicata* spicules corrodes away completely, but a difference is observable in the behaviour of the different types of ray. The slender monaxons and the basal rays of the tri- and quadriradiates are more slowly attacked and display only a slight corrosion when the oscular rays, gastral rays, and curved monaxons have completely lost their calcite. Such a difference was unnoticed by Bütschli, who used the large monaxons of *Leucandra aspera* almost exclusively for his research, and also by v. Ebner (1887), who used the regular triradiates and the monaxons of *Ascandra falcata*. It is presumably to be correlated with the different orientation of the optic axis in these rays (Jones, 1954), or with differences in the purity of the calcite (see later); it seems unlikely that the spicule sheath varies in its protective ability according to the type of ray enclosed, especially when the different types belong to the same multi-rayed spicule.

As the calcite corrodes, a sharply defined 'sheath' becomes visible at the original surface of the rays and this thickens inwards so that only a small space can be seen between its inner surface and the calcite remnant. Eventually this remnant disappears, sometimes breaking up into small 'islands' in the oscular rays before vanishing completely. The corrosion is not uniform all over the spicule surface, for as v. Ebner (1887) noticed in his experiments, the areas transverse to the optic axis are preferentially attacked. This is particularly noticeable when the action is less vigorous, as when 1% potash solution is employed. In this solution the surfaces parallel to the optic axis remain

FIG. 1 (plate). Corrosion by potash solution.

A, triradiate with almost completely corroded oscular rays.

B, enlargement of the basal ray tip of A, showing the fork caused by corrosion on mainly the gastral and dermal (o.a.-transverse) surfaces.

C, quadriradiate with uneven 'front' and 'back' oscular ray edges caused by corrosion on the o.a.-transverse surfaces.

D, quadriradiate seen in side-view, with roughened o.a.-transverse edges on the gastral ray.

E, much-corroded triradiate with the calcite reduced to a pair of splinters at the proximal end of the basal ray.

F, corroded quadriradiate in side-view with no calcite remaining in the gastral ray. Note the absence of forking at the basal ray tip in this view, and the retreat of the calcite from the gastral and dermal surfaces.

G, lance-headed monaxon with uneven o.a.-transverse edges on the calcite remnant.

H, the potash-isolated 'sheath' of a lance-headed monaxon. Note the transverse striations on the deposit.

I, the potash-isolated 'sheath' of a triradiate, again showing the discontinuity of the deposit. Note the concentration of scales within the 'sheath' at the central junction.

J, a 'sheath' isolated by means of $\frac{1}{2}$ % potash solution.

Spicules A-G were mounted in DPX and photographed by plane-polarized light, the plane having been rotated until the calcite was most distinct; H-J were in air.

A, B, E, F, and G were 18 hours in 5% potash at 58° C: C and D, 46 hours in 1% potash at about 20° C: H and I, 19 hours in 10% potash at 58° C: J, 46 hours in $\frac{1}{2}$ % potash at about 20° C.

In each photomicrograph the continuous line represents 50 μ ; the broken line, 25 μ .

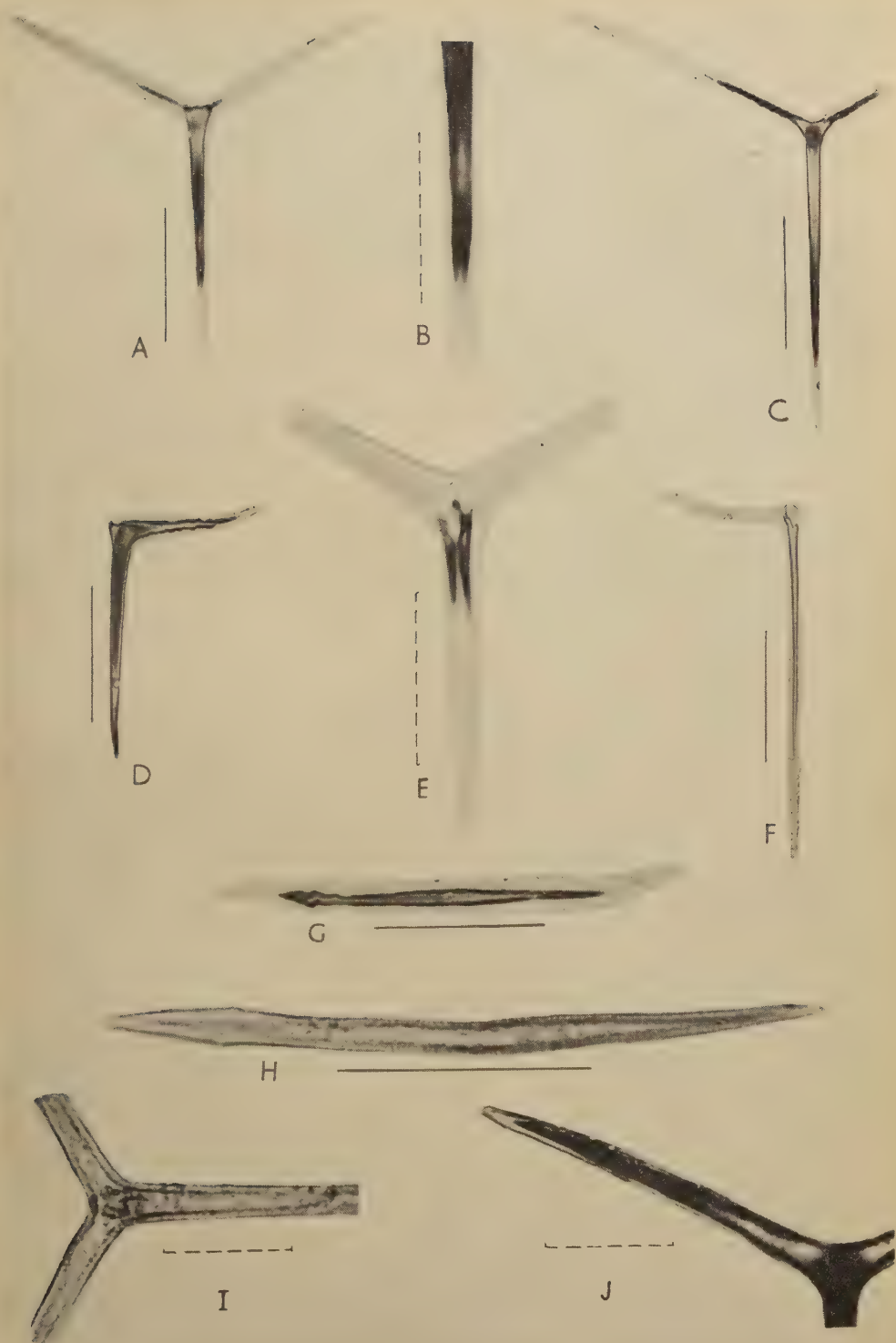


FIG. 1
W. C. JONES

smooth, but the o.a.-transverse surfaces appear roughened when seen in profile on the oscular rays, gastral rays, and curved monaxons (fig. 1, C, D, and G); while in surface view on the basal rays they develop pits along their centre and the calcite tip becomes forked (fig. 1, A and B). At a later stage the basal ray calcite divides into two proximal splinters due to this selective corrosion (fig. 1, E), appearing however single in side view (fig. 1, F). Faint striations, roughly in the direction of the basal ray, may furthermore be seen lying across the oscular rays, and these apparently are corrosion pits extending through the ray centre between the opposite o.a.-transverse surfaces. Similar striations parallel to the optic axis may also be seen in curved monaxons. The slender monaxons, however, diminish uniformly in length and thickness; their optic axis coincides with the spicule length and they are sharply pointed at each end, so that the o.a.-transverse faces will not be large in area.

The time taken for the complete dissolution of the calcite depends on the temperature and concentration of the potash solution, but a typical result for a 10% solution is $8\frac{1}{2}$ hours at 58° C, and for a 5% solution about $2\frac{1}{2}$ days at approximately 20° C. Prolonged immersion in the former solution yields a thicker internal deposit, but otherwise there is little difference between the 'sheaths' produced by the different strengths of potash.

The resulting spicule 'sheath' when examined in air is practically colourless, but with a faint bluish (sometimes greenish-yellow) tinge, probably caused by residual chromatic aberration. In optical section it exhibits a smooth outer edge and a less distinct, roughened inner edge, and it appears faintly striated, the striations lying transverse to the 'sheath' (fig. 1, H and I). These striations suggest that the 'sheath' consists of closely-set blocks, and they are particularly evident on the 'sheaths' of oscular rays and curved monaxons. The basal rays, on the other hand, have thinner 'sheaths' at the o.a.-parallel surfaces, but exhibit a granular deposit in a strip along the centre of their gastral and dermal surfaces, which appears as a thick 'sheath' on each edge when the basal ray is examined in side view. The observations are consistent with the assumption that as the calcite recedes a deposit is laid down in the form of closely-compressed 'pinnacles' on the inside of the true spicule sheath, and builds up mainly from the o.a.-transverse surfaces in the regions of most active corrosion.

Just within the 'sheath' are found some isolated flattened scales of deposit, particularly in the oscular rays and curved monaxons. The scales are especially distinct at the central junction of the tri- and quadriradiates (fig. 1, I), where they are concentrated to form a three-cornered structure which appears to be separated from the outer 'sheath' when seen in both side and surface views of the spicule, though it is not completely free. The 'sheaths' are brittle and fracture easily at the touch of a needle, and it is possible to remove a part, leaving a corner of this central structure projecting through the opening. Similarly the 'sheath' above or below this structure can be broken away, though it has not been found possible to isolate the latter. It seems probable that the scales are associated with the organic axial filament, which is first

formed in triradiates as a central triangle upon which the birefringent calcite crystallizes (Minchin, 1898; Minchin and Reid, 1908).

Between crossed nicols the discontinuity of the 'sheath' becomes more pronounced, particularly with the oscular rays and curved monaxons. Here the 'sheath' in optical section appears as a row of pale yellow blobs, which darken as the 'sheath' of each ray is rotated into the positions parallel or perpendicular to the plane of polarized light. Sometimes a very fine yellow streak lies outside and separated from the row of blobs and seems to mark the outer edge of the true sheath, while on the inside the scales glow faintly yellow and darken roughly at the same time as the adjacent blobs, though the correspondence is often not exact. The basal ray 'sheath' also is birefringent in optical section, generally appearing in the positions of maximum brightness as a bright orange streak, or, especially when the spicule is examined in side view, as a row of bright yellow blobs. In surface view are also seen scattered points of light on the dermal and gastral surfaces of the basal ray. In DPX or Canada balsam (refractive index, 1.53) all the birefringence phenomena disappear, but they are still present, though weaker, when water is the ambient fluid; the birefringence does not therefore seem to be intrinsic.

When mounted in DPX or balsam the 'sheath' is indistinct and hence has a refractive index close to 1.5. When it has not been thoroughly dried it exhibits a pattern of small refringent beads on its inner surface, the beads being usually restricted to the o.a.-transverse regions. They are tiny droplets of water and appear to be sited between the bases of the 'pinnacles' of deposit; they vanish after complete dehydration.

Finally, when examined in air the potash-isolated 'sheaths' show a series of fine, evenly spaced, broken lines parallel to their outer edge and arranged across the ray surface. These are diffraction fringes since they extend outside the ray boundaries when the focus is lowered, and they disappear when a No. 1 coverslip is laid above the 'sheath'.

Proof that the potash-isolated 'sheath' is an artifact

There can be no doubt that the 'sheath' isolated by potash solution is not a property of the normal spicule. As described above, the spicules dissolve in distilled water (pH 6.5), leaving only a very thin, partially contracted sheath, whereas in the same liquid the thick potash-isolated 'sheaths' persist for days longer. Furthermore spicules corroded by distilled water (pH 6.5) until much reduced in size do not exhibit such 'sheaths', but yield them at their reduced outline when immersed in potash solution. Since these 'sheaths' are rigid and non-contractile in distilled water, they cannot have bounded the original surface of the spicule, but must have been produced at the present spicule surface. Young spicules with basal rays no more than 25μ long likewise produce the rigid 'sheaths', yet if they were a property of the normal spicule, growth would have been impossible. The thickness and flakiness of the 'sheaths' also preclude the possibility of their presence on the untreated spicules, as does the extension of the 'sheath' across the ends of broken rays.

The nature of the potash-isolated 'sheath'

In Bütschli's original paper (1901) the suggestion is made that the inorganic constituent of the 'sheath' is probably calcium carbonate, but in his later paper (1907) he states that calcium hydroxide is precipitated when calcium carbonate and strong potash react together, though he makes no assertion here about the nature of the 'sheath'. However, such a precipitation would imply that within the sheath the product $[Ca^{++}].[OH^{-}]^2$ equals the solubility product of calcium hydroxide. Now the maximum value that one would expect for $[Ca^{++}]$ would be the square root of the solubility product of calcite ($CaCO_3$), assuming that $[CO_3^{--}]$ does not fall appreciably below $[Ca^{++}]$ by the recombination of CO_3^{--} ions with any other cations. Taking values derived from Johnston and Williamson (1916) for the solubility products of calcium hydroxide and calcium carbonate, namely 4×10^{-6} ($16^\circ C$) for the former, and 0.98×10^{-8} ($16^\circ C$) for the latter, and assuming a degree of ionization of 0.8 for the potash solution, then

$$\frac{4 \times 10^{-6}}{\sqrt{(0.98 \times 10^{-8})}} = [OH]^2 = \left[\frac{0.8 \times C}{56} \right]^2,$$

where C is the concentration of potash in g per litre, below which calcium hydroxide could not be precipitated, whence

$$C = 14 \text{ g per litre, or } 1.4\%.$$

This affords a method of testing whether calcium hydroxide is precipitated in the formation of the 'sheath', for potash solutions of concentration less than 1.4% should not produce a deposit at normal room temperature. However, slides placed in potash solution of only $\frac{1}{2}\%$ at 16.5 – $22.5^\circ C$ form well-defined 'sheaths' (implying a Ca^{++} concentration 10 times greater than the maximum assumed above), which are similar to those given by 1, 1.5, 2, 5, and 10% solutions (fig. 1, J), though the rate at which the calcite corrodes is proportional to the concentration used and is very slow in the $\frac{1}{2}\%$ solution. The residue is therefore probably a compound other than calcium hydroxide, possibly a combination of this with a carbonate. However, Bütschli's diagram (1907, fig. VIA, p. 313) shows hexagonal crystals of calcium hydroxide mounted on a spicule 'sheath' with the corresponding sides all parallel, and though Bütschli does not comment on this oriented overgrowth, it is possible that the organic sheath has oriented the crystals by chemical combination with them.

It is not possible to prove the presence or absence of carbonate by treating the 'sheaths' with dilute hydrochloric acid, since the spicule calcite can dissolve without forming bubbles of carbon dioxide. In any case it would be more practicable to use giant calcareous spicules from another species of sponge for a chemical investigation of the nature of the true and potash-isolated sheaths.

The action of hydrochloric acid on normal spicules

If dilute (0.2%) hydrochloric acid is drawn under a coverslip on a slide of spicules isolated by means of potash or potassium nitrate solution, the spicule calcite quite rapidly dissolves, revealing the thin sheath, which varies somewhat in thickness from spicule to spicule and often bears localized swellings or foreign matter on its outer surface. The sheath usually contracts immediately the contained calcite has receded, the amount of contraction depending on the sheath thickness, though usually the empty envelope has a diameter about half that of the original ray. Parts (particularly of the basal ray) may even collapse completely to form an apparently solid strand. However, the sheath possesses some degree of rigidity, enough to support the weight of uncorroded calcite on the sheath tips and to maintain the spicule shape in some cases, though its flexibility is revealed by the swaying of freely projecting parts with movements of the ambient solution.

When 10% followed by concentrated hydrochloric acid is applied to these sheaths, they become more transparent and much less distinct, with sometimes an increase in contraction. Their fragility also increases, the sheaths usually crumpling or breaking as the acid is drawn across them. Some residue seems to be stable in the concentrated acid, however, particularly as some sheaths show no signs of change after 2 hours. When applied directly to the spicules, the stronger acid solutions dissolve out the calcite rapidly and cause the complete collapse of the sheath to a twisted wisp. The strong hydrochloric acid thus seems to soften the sheath, perhaps by dissolving out a component, which could be chitin, though this has not been found to be generally present in the Porifera (Richards, 1951).

The calcite corrodes away smoothly without the evolution of bubbles of carbon dioxide unless strong acid is used, when the bubbles form at the surface of debris on the slide, or sometimes inside the sheath, which contracts around them. No tendency for the rays to become paired by uneven corrosion is observed and the last calcite to vanish is the part at the spicule centre. No trace of an axial filament has ever been seen (agreeing with Bütschli's observations), but Minchin and Reid (1908) have pointed out the difficulty of demonstrating its presence in spicules of *Leucosolenia complicata*, and in any case it cannot be detected when spicules of *L. coriacea* are treated with 0.2% hydrochloric acid, though these are said to possess well-defined axial filaments.

The action of hydrochloric acid on potash-isolated 'sheaths'

When 0.2% hydrochloric acid is repeatedly applied to the 'sheaths' isolated by 10% potash solution, the calcite remnants (if any still exist) first disappear and the 'sheath' gradually becomes more transparent and colourless. Progressively it gets thinner and clearer with the dissolution of the inorganic deposit on the inside, and eventually the true sheath contracts to form an uneven, distorted outline of the original spicule. Sometimes it snaps across at some point when the longitudinal contraction between the points of attach-

ment becomes excessive. As above, the addition of strong acid results in the sheath becoming less distinct and more fragile; it is easily distorted by movements of the solution.

The potash-isolated 'sheaths' of spicules much reduced in size by prior corrosion likewise reveal a true sheath when treated with dilute hydrochloric acid. This sheath had presumably collapsed around the corroding spicule ray before becoming supported by the potash-produced deposit. The latter is undoubtedly responsible for the rigidity of the 'sheath', which is shown by the preservation of the spicule form despite the process of washing and drying.

Corrosion of spicules bearing crystals

Some spicules bearing crystals (Jones, 1955) and mounted in old glycerine jelly revealed after about 15 hours that their ray calcite had corroded away from the spicule sheaths upon which were perched the uncorroded crystals (fig. 2, A-D). The same result, namely, that the ray calcite always corrodes at a much faster rate than the crystals, is also obtained when such corroding media as acid alcohol, 10% potash solution, carbonic acid, or distilled water are used. For example, in the potash solution the crystals have dissolved only after from 3 to 4 days (58° C), whereas the ray calcite vanishes in about half a day. The difference is presumably due to a difference in the purity between the ray calcite and the crystals; the latter are pure since they were crystallized from calcium bicarbonate solution, whereas the former probably contain traces of magnesium, sodium, sulphate, and possibly water, as do spicules of *Leucandra aspera* (v. Ebner, 1887).

The crystals remaining on the sheath have flat inner surfaces and seem to have crystallized directly on the latter, though there is still the possibility that they were merged with the underlying calcite through perforations in the sheath. When washed and dried the true or potash-isolated sheaths with crystals can easily be teased away, while the process of making a permanent preparation often removes an entire sheath and its attached crystals from a ray remnant. The crystals then normally retain their original crystallographic orientation with respect to the ray axes, though the distortion or breakage which the sheath usually suffers when separated from the supporting calcite sometimes throws some of the crystals out of orientation, unless they are fused laterally together. There is no doubt that the crystals are firmly attached to a moderately rigid envelope, even when the sheath has not been isolated by potash, but possibly some of the rigidity arises from the plugs of calcite that presumably fill the perforations in the true sheath when the crystals have been formed (see later).

The glycerine jelly usually corrodes the ray calcite from principally the o.a.-transverse surfaces, so that these become roughened or bear numerous parallel projections, each in line with the optic axis. Parallel striations across the face of the oscular rays also become visible as with potash corrosion, while along the centre of each ray the calcite appears more transparent

presumably through corrosion in the region of the axial filament. Eventually the calcite may be only represented by rodlets arranged like the rungs of a ladder, each rung tending to be parallel with the optic axis. The apparent selectivity of the corrosion is presumably due once more to variations in the purity of the calcite throughout the ray. The basal ray again seems more stable than the oscular rays, as with potash corrosion.

When spicules without crystals are mounted in glycerine jelly they corrode much more slowly than the spicules bearing crystals. The effect is due to the prior corrosion which the latter have had to suffer before the crystals could form on their surface, for if normal spicules are immersed in carbonic acid solution (pH 4.0) for 5 minutes before drying and mounting in glycerine jelly, their rate of corrosion increases markedly. The ability of the glycerine jelly to corrode wanes rapidly; after one year slides of spicules bearing crystals in this medium may still exhibit uncorroded crystals and may not even have lost all the calcite from the basal rays. The corrosion is also much less evident in freshly-bought glycerine jelly.

When spicules bearing crystals are corroded by 10% potash the crystals in time dissolve and leave a residue marking their original outline. These residues vanish in dilute hydrochloric acid without leaving an insoluble component.

Crystallization experiments with isolated sheaths

The corrosion experiments in the preceding section indicate that the crystals may have crystallized directly on the spicule sheath, much as the calcite is presumed to crystallize on the axial filament by a process of oriented overgrowth (Jones, 1954). Attempts have therefore been made to crystallize calcite on the sheath after the ray calcite has been removed by prior corrosion. Two principal methods have been adopted. In the first the spicules were corroded by acid alcohol or carbonic acid solution until reduced in size, and were then washed and treated with the crystallizing solution with or without prior drying. In neither case have crystals ever convincingly been obtained unattached to calcite remnants. The crystals always lie on the calcite remnant, even when

FIG. 2 (plate). Corrosion by glycerine jelly of spicules bearing crystals (A–E).

A, corroded lance-headed monaxon with uncorroded crystals attached to the spicule sheath.
B, early stage in the corrosion of a quadriradiate. The o.a.-transverse edges of the oscular rays are uneven.

C, later stage for a triradiate; little calcite remains within the oscular ray sheath and crystal rows. The basal ray calcite has receded about 50μ from the original ray tip.

D and E, two stages in the corrosion of quadriradiates seen in side-view. Little calcite remains within the sheath of E.

F, attempt to crystallize calcite directly upon the potash-isolated 'sheath'. Crystals have only formed within the 'sheath' and do not have similarly oriented optic axes (note the differences in distinctness). The remnant of the basal ray calcite can be distinguished, indicating that the original spicule calcite had not been completely corroded away, and that there was therefore the probability of the crystals having overgrown on calcite remnants.

All the spicules were photographed in plane-polarized light, the plane having been rotated until the spicule calcite was most distinct.

The line in each photomicrograph represents 50μ .

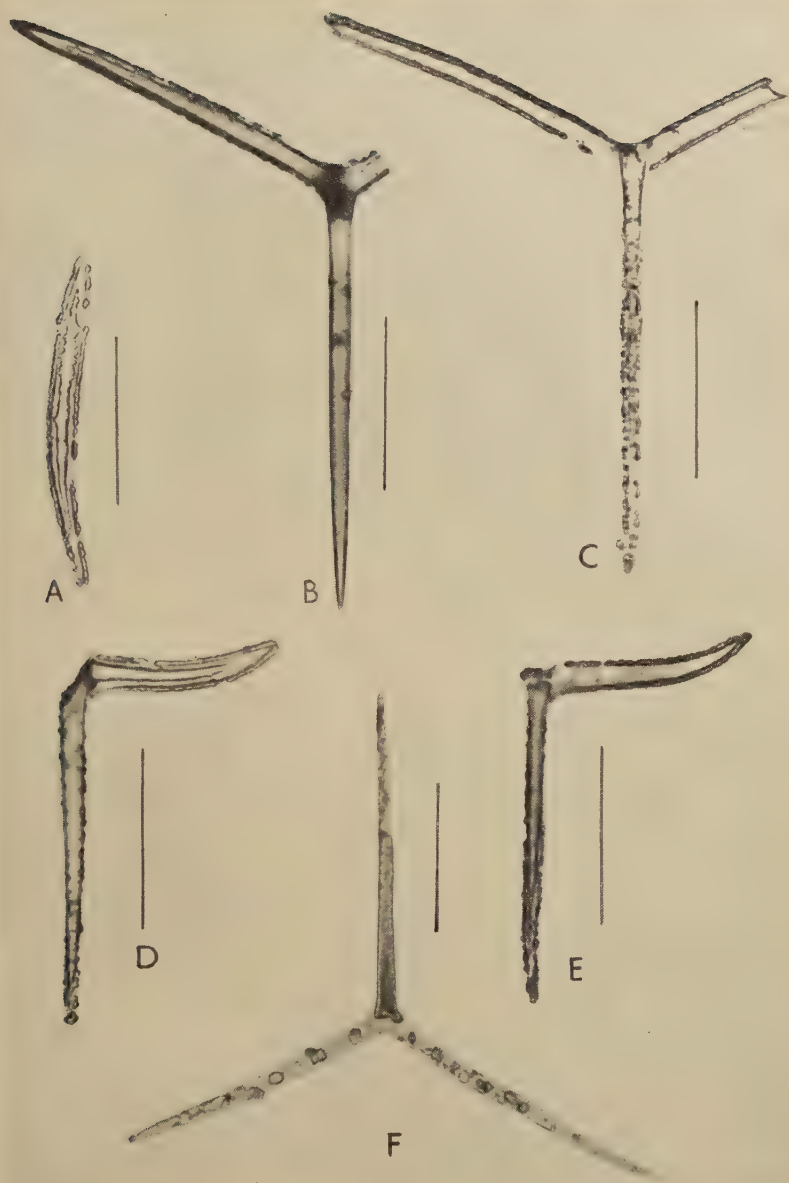


FIG. 2
W. C. JONES

this can clearly be seen to be within the spicule sheath. Their pattern may be like that normally obtained with potash-isolated spicules (Jones, 1955), though often the crystals cover the surfaces of the remnant. When they appear to be separated from the calcite remnants, the space in between gives a faint glow between crossed nicols, and hence is probably filled with calcite traces.

In the second method potash-isolated 'sheaths' were used, but again there was never any indication of the formation of oriented crystals on the outer surface, though this is probably the normal surface of the spicule. At times large crystals inside the 'sheath' have been obtained (fig. 2, F), but these are dis-oriented and appear to have crystallized on calcite remnants, especially as the basal ray calcite was not completely dissolved. The distorted sheaths left after the removal of the internal deposit by means of dilute acid likewise do not form sites for crystallization and thus there is no evidence that calcite can form oriented overgrowths directly on the sheath.

In some early crystallization experiments it was observed that the crystals were not properly attached to the surface of the ray calcite, but seemed to be connected by narrow peg-like bridges, or to be supported only by the sheath. It is now known that this was due to the use of two successive lots of crystallizing solution of pH about 7.0 at the start. The first lot had begun to crystallize before the addition of the second, which then corroded the ray calcite slightly while its pH was rising to the crystallizing level (about pH 8.0); when crystallization recommenced it took place on the now peg-like or separated crystals, rather than on the receded ray calcite. It has already been recorded that crystallization is less easily accomplished on the spicule calcite than on the growing crystals, possibly on account of the intervention of the sheath (Jones, 1955).

It is thus concluded that no convincing evidence exists favouring an oriented overgrowth of the calcite crystals on the spicule sheath. It is much more likely that the crystals are oriented by crystallizing on the ray calcite through perforations in the sheath.

I wish to record my indebtedness to Professor F. W. Rogers Brambell and to Dr. D. J. Crisp (of the Marine Biological Station, Menai Bridge, Anglesey) for their encouragement and for helpful discussions. Dr. C. F. A. Pantin kindly read and criticized the manuscript.

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Refractometry of Living Cells

Part III. Technical and Optical Methods

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SUMMARY

Full details are given in this paper for carrying out the measurement of the refractive index of living cells by the method already discussed in Parts I and II of this series (Barer and Joseph, 1954, 1955). The method of preparing and storing immersion media is described as well as the dialysis technique used for adjusting pH, and in certain cases salt concentration. Methods of measurement applicable to single cells and to cell populations are discussed. It is shown that the numerical results can be used to obtain the concentration of solids, concentration of water, total wet mass and density, and concentrations in terms of wet weight of tissue. The underlying optical principles are discussed in some detail.

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TECHNICAL METHODS

THE basic principles and a discussion of a number of possible immersion media have already been given in Parts I and II of this series (Barer and Joseph, 1954, 1955). Bovine plasma albumin, fraction V (Armour Laboratories), has been found to be the most generally useful medium, and [Quarterly Journal of Microscopical Science, Vol. 96, part 4, pp. 423-447, December 1955.]

the remarks which follow should be taken as applying to it, though many of the techniques described have also been used for other substances.

Method of solution

The simplest way of making a stock solution of bovine plasma albumin, fraction V, is to add the dried powder *little by little* to water or a salt solution. This should be done in a small but relatively wide beaker or cylindrical tube. Generally speaking, we have found it convenient to make up about 5 ml of solution at a time, and have used corked cylinders 25 mm in diameter and 50 mm high. The dried powder is extremely light and bulky and it is scarcely possible to make a concentrated solution by adding water to a weighed quantity of powder. If this is attempted, a very sticky and frothy mass is produced which clings to the sides of the containing vessel and to the stirring rod. If, on the other hand, the powder is added little by little to an excess of solvent, and is well stirred with a glass rod, relatively little frothing should occur. After each addition of powder the solution becomes very cloudy, but clears after standing for a few minutes when more powder can be added. This process is repeated until the required concentration, as judged by the refractometer reading, is attained. It should be possible to make solutions of about 40% concentration in less than half an hour, but the viscosity seems to rise fairly rapidly between 40 and 50% and solution becomes progressively slower. When first made, the solutions nearly always have a layer of froth on their upper surfaces, but this usually disappears after standing for a few hours, preferably in a refrigerator. If, however, it is essential to clarify the solution rapidly, and at the same time to remove all froth, this can be done quite easily by centrifuging at moderate speed.

Avoidance of frothing

Proteins can be denatured by excessive frothing, and mechanical agitation other than gentle stirring with a glass rod should be avoided. We have carried out some experiments with DCA silicone anti-foam. This is obtainable as a thin paste and a very minute smear placed on the glass vessel or stirring rod substantially reduces frothing. The material appears to be biologically inert and without any obvious action on the protein, but insufficient work has been carried out with it as yet to be certain that it is suitable for use with all types of material. In any case, with increasing experience in the making up of protein solutions, frothing becomes an unimportant problem.

Keeping properties

Solutions should be kept in small vessels closed by a rubber bung or cork. Glass stoppers should not be used because they are liable to become firmly wedged by thin films of dried protein which may be deposited accidentally at the mouth of the vessel. The stock solution should be kept in a refrigerator. Under these conditions bacterial contamination is rare, but in our laboratory yeasts and fungi invariably appear after about one week. For this reason only

small quantities of concentrated stock solutions should be made up at a time. It is possible that the addition of small amounts of antibiotics may allow solutions to be kept for much longer periods. We have not, however, carried out any experiments with such substances.

Adjustment of concentration

The stock solution may be diluted with water or an appropriate saline solution to make up any desired concentration. For most work only a very small volume of solution of any one concentration (say about 0.5–1 ml) is needed. Such solutions are mixed in small, flat-bottomed cylindrical glass tubes (12 mm diameter, 30 mm high). The concentration is estimated by means of a refractometer as described below. Thorough mixing of the concentrated stock solution with the diluting fluid is essential. If mixing is incomplete, different readings of refractive index may be obtained from different samples of the mixture, and in some cases it may be difficult to obtain a sharp dividing line in the field of view of the refractometer. A simple method of mixing is to suck the solution up and down a number of times in a clean glass pipette fitted with a rubber teat. This should not cause much frothing. When working with very small volumes of solution, it is essential to avoid evaporation by keeping the tubes well corked. The concentration should be checked shortly before use and one should not rely on values obtained on the previous day, for example.

Refractometry of solutions

Any reasonably accurate refractometer can be used for the estimation of protein concentration. The type we have found particularly suitable, however, is the small hand refractometer as used in industry for estimating the percentage of total solids. They are sometimes known as 'sugar refractometers' and their scales are graduated in terms of percentage sugar concentration. Instruments of this type are made by several manufacturers, including Bellingham and Stanley, Bausch and Lomb, and Zeiss. They work on the critical angle principle and only require a very small drop of liquid, which is placed as a thin film between two small glass prisms. Although the scale is calibrated in terms of sugar concentration, a conversion table, giving refractive indices, is provided. Instruments which cover the range 0–50% sugar (corresponding to refractive index 1.333–1.420) are the most useful. The graduation interval is 1% sugar, corresponding to refractive index steps of about 0.0015. The makers usually claim that estimations can be made to one-tenth of a scale division. Even if this is perhaps a little optimistic, it should certainly be possible to estimate to within one-fifth of a division, corresponding to refractive index differences of about 0.0003 or 0.2% of solids. This degree of accuracy is more than adequate for most purposes. The sugar refractometer is compact, inexpensive, sufficiently accurate, and very rapid in use.

No matter which type of refractometer is used, it is essential to wash off any protein solution immediately after use. If the solution is left to dry, a

hard crust forms which is extremely difficult to remove. The results may be disastrous in the case of critical-angle refractometers, in which the two prisms may become almost inseparably adherent to one another.

Dialysis and adjustment of pH

For most purposes the protein powder as supplied by the manufacturers can be dissolved in the appropriate salt medium and used without further treatment. In some cases, however, it may be necessary to remove even the small amount of salt normally present, or to change the pH of the medium. The salt content can be reduced by dialysis against distilled water. Alteration of pH is rather more complicated; it can be effected by the addition of sodium hydroxide, but there is some risk of denaturation. It appeared better, therefore, to dialyse the protein solution against a suitable buffer. If necessary the pH-adjusted protein solution can be equilibrated finally by dialysis against a 'physiological' medium such as a buffered Ringer solution. The latter procedure is probably the most satisfactory on theoretical grounds since there is no guarantee that the pH or osmotic properties of a solution containing protein, when in contact with a cell surface through which some exchange of ions can take place, will be the same as those shown by a glass electrode or by freezing-point determinations. The ion exchange will be further affected by the presence of protein on each side of the cell surface, so that a complicated Donnan equilibrium will be set up. Conditions of this sort led us to prefer a 'biological' definition of tonicity, based on cell volume, to any definition based on physical properties alone (see Part II, Barer and Joseph, 1955). If, however, the cell can be regarded as being in equilibrium with a 'physiological' medium, a protein solution equilibrated against the latter should also be in equilibrium with the cell.

Dialysis was carried out in tubes of synthetic cellulose dialysis casing. The solution of protein or other substance was placed in the tube which was securely tied at both ends with strong thread. Most of the substances of high molecular weight that were investigated exerted a considerable colloidal osmotic pressure, so that the tubes underwent considerable swelling. Although the tubes very rarely burst during dialysis, the contents became greatly diluted by uptake of water. In order to reduce this dilution the cellulose tube was surrounded by a 'stocking' of Swiss silk bolting cloth, very slightly bigger than the inner cellulose tube. The ends of the outer tube were also tied with strong thread. Even with these precautions considerable dilution occurred, and in order to obtain a 20% protein solution it was usually necessary to start with a concentration of between 30 and 40%. The outer stocking could withstand the pressure developed by most of the substances investigated, but with polyvinylpyrrolidone the pressure was so great that at least two outer stockings had to be used.

If the object of dialysis was merely to reduce the salt content or to remove substances of low molecular weight, the material was dissolved in distilled water and dialysed against distilled water. The cellulose tube and its outer

stocking were placed in a large vessel containing a volume of water at least 10 times as great as that of the solution. The vessel was placed in a refrigerator and the distilled water changed at about 6-hourly intervals. At least three changes were necessary and as a rule dialysis was continued for about 48 hours. At the end of this time the tube was removed and its contents emptied into a covered flask. For some purposes, when a fairly low concentration of salt-free medium was required, the dialysed solution could be used at once, but as a rule it was found more convenient to freeze-dry the material, which was then stored as a dried powder in a stoppered bottle kept in a refrigerator. Solutions of any desired concentration could then be made up in water or a suitable salt medium. This procedure has been adopted with many substances, particularly commercial egg albumin and acacia gum.

In order to change the pH of the material, a concentrated solution was made in a suitable buffer, placed in a cellulose tube with outer stocking as already described and then dialysed against the same buffer. For most of our work we have used a sodium phosphate buffer containing 11.36 g Na_2HPO_4 , 3.12 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, and water to 1 litre. The pH of this is 7.4. At least three changes of buffer at 6-hourly intervals were required. If it was desired to make the final solution as salt-free as possible, further dialysis was carried out against a similar buffer solution diluted 10 times with water and finally against distilled water. The pH of the final material was usually between 7.0 and 7.2. If, on the other hand, it was desired to equilibrate the medium against a physiological salt solution, the final dialysis was carried out against a buffered Ringer-Locke solution (0.54 M NaCl 270 ml, 0.54 M KCl 5 ml, 0.36 M CaCl_2 5 ml, phosphate buffer as above 12 ml, distilled water to 1 litre). It should be pointed out that media which are not salt-free cannot be concentrated by evaporation or freezing-drying without changing the tonicity. If very concentrated solutions are required, it is therefore best to dialyse against distilled water and to freeze-dry. The tonicity can then be adjusted by dissolving the frozen-dried material in the correct salt medium.

REFRACTOMETRY OF CELLS

There are many possible types of technique which can be used in cell refractometry. Perhaps the first thing to decide is whether one wishes to adopt a 'single cell' or a 'cell population' method. In the former, detailed measurements are carried out on one individual cell which has to be kept under constant observation. In the latter, the distribution of refractive index or solid concentration among different members of a cell population is measured. Accurate measurements on any individual cell are not necessary in this case, and, provided sufficient cells are observed, the same group of cells need not be kept constantly in view.

Single cell methods

The problem of refractometry of single cells is to keep the chosen cell stationary while the refractive index of the medium is being changed. In the

case of cells which are fairly firmly attached to the slide or the coverslip, such as many cells in tissue culture or amoeboid cells, the simplest method is to irrigate the preparation with protein solution. A drop of solution is placed by means of a pipette at the edge of the coverslip and is drawn through by a piece of filter paper placed in contact with the liquid at the opposite edge of the coverslip. This process may have to be repeated several times in order to ensure complete replacement of the original fluid by the new medium. Care must be taken to ensure that the preparation does not become sealed off by a layer of dried protein, though this is only likely to occur when very concentrated solutions are used. In any case the method is not very suitable when concentrated solutions are required as the latter cannot be run through the preparation very easily because of their high viscosity. An obvious criticism of this method is that unless there is proper mixing or complete replacement of the fluid originally present, the true value of the refractive index may not be obtained. In practice, however, such difficulties rarely arise and the values obtained by irrigation agree extremely well with those obtained in other ways.

The irrigation technique has been greatly simplified and improved by the development of a special chamber which allows the medium to be changed smoothly at a controlled rate (Dick, 1955). This also reduces the risk of mechanical damage.

In the case of cells which are not firmly anchored and are therefore liable to be washed away by the stream of protein solution, simple expedients, such as the use of fine threads, hairs, or cotton wool, can be tried. These usually prevent many of the cells from travelling far from their original positions, and if the protein is run in carefully, an individual cell can usually be followed under the microscope.

The irrigation technique is particularly valuable for purely observational work. It is often most instructive to observe the sequence of changes in the appearance of a cell as the refractive index of the mounting medium changes slowly and continuously. If the cell is mounted in water or a saline solution, for example, and observed by positive phase-contrast while a sufficiently concentrated protein solution is run in slowly, various regions of the cell will gradually lighten, then disappear, and finally reappear with reversed contrast. If now the protein is washed out by water or saline, the series of events will be reversed. In this way regions of different refractive index can be distinguished and an excellent differentiation of internal structure may be obtained. The method can in fact be regarded as a type of 'optical dissection' (see Barer, 1954 *b*). Although in most cases observations in media of several different refractive indices have to be carried out, nevertheless in certain cases in which the refractive index of the cell is undergoing functional changes, it is often useful to observe the cell in a medium of fixed chosen refractive index. Thus, for example, changes in refractive index occur during the movement of amoebae, in cell division, and during germination of spores. (A series of photomicrographs showing cell division in a protein medium is given in

fig. 3 of Part II.) If such cells are immersed in a medium of a given refractive index, those regions of the cytoplasm with indices either above or below that of the medium can be distinguished. In some cases cells can actually be grown in the protein medium and refractive index changes at different stages of the life-cycle of an organism can be followed. This technique has been applied especially in the case of fungal spores, which usually grow quite well in sealed preparations without any special provisions for access of oxygen. If air is required, the cells can be grown in hanging drop protein preparations, provided that precautions are taken to avoid evaporation. The protein drop can be surrounded by a liquid paraffin drop for this purpose.

Cell population methods

In many cases one may be less concerned with the exact refractive indices of different regions of a single cell than with the variation in refractive index among many cells of the same type. This is particularly the case with red blood-cells and bacteria, for example. Strictly speaking, of course, both types of information are necessary because all cells are subject to biological variation and the refractive index of part of a single cell has relatively little meaning unless the degree of variability among cells of the same type is also known. The cell population technique described here was developed for haematological work (Barer, Howie, Ross, and Tkaczyk, 1953), but can be applied to almost any type of cell.

Ideally, perhaps, we should like to be able to plot a distribution curve showing as ordinate the number of cells having a given refractive index (i.e. containing a certain solid concentration) and with the refractive index or solid concentration as abscissa. We could, in principle, take a known number of cells in a counting chamber, immerse them in a medium of a certain refractive index, and then find the number of cells which have disappeared by counting the number still visible. This would be rather difficult in practice, however; it would be almost impossible to keep the number of cells constant while changing the immersion medium. Also, it is very difficult to decide exactly when a cell or part of a cell has disappeared without making detailed observations on each individual cell. Fortunately it is rarely necessary to know the absolute number of cells. A knowledge of the *relative* number containing a given concentration usually suffices. In order to avoid having to count 'invisible' cells, we can instead count the total number of dark and bright cells in a given field of view. The cells so near the match-point that it is impossible to decide whether they are dark or bright are counted separately and divided equally between dark and bright. Since the number of such cells is generally small compared with the total number in the field of view, the error in this procedure is small. After counting a number of random fields in this way for each concentration of immersion medium, we can plot a curve showing the proportion of cells having more or less than a given refractive index (i.e. containing more or less than a given solid concentration). In general the result is a sigmoid curve known as an integrated distribution curve or

Galton's ogive. The relationship between this curve and the more usual type of distribution curve is shown in fig. 1. In fig. 1, A the number (n) of cells containing a given concentration is plotted against the concentration (C). In fig. 1, B the percentage (p) of cells containing more than a given concentration is plotted against C . Now in A the total number of cells N is the area

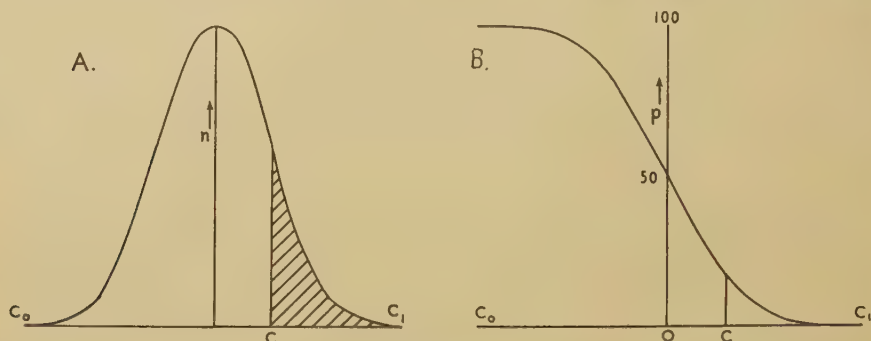


FIG. 1. The relationship between the normal distribution curve A and the integrated distribution curve B. In the former the number n of cells containing a given concentration C is shown.

In B the percentage p of cells containing more than a given concentration C is shown.

under the curve, i.e. $N = \int_{c_0}^{c_1} n dC$. The number of cells containing more than a given concentration C is shown by the shaded area which is equal to $\int_C^{c_1} n dC$. Hence the percentage of cells containing more than a given concentration is

$$p = \frac{\int_C^{c_1} n dC}{\int_{c_0}^{c_1} n dC} \times 100$$

$$= K \int_C^{c_1} n dC$$

where K is a constant ($= 100/N$). Thus the ordinate p of curve B is proportional to the shaded area in curve A. The distribution curve A can in principle be derived from B by differentiating the latter, i.e. by plotting the slope of curve B against C . In practice, however, it is not always possible to measure the slopes with sufficient accuracy. The integrated distribution curve gives essentially the same statistical information as the ordinary distribution curve: it is simply a matter of convenience which is used. It is sometimes useful to plot the integrated curve on 'probability paper'. Such a plot will be linear if the distribution is a 'normal' one.

The exact method of determining the integrated distribution curve depends on the type of material used and the degree of accuracy considered

desirable. For haematological work counts were made on 200 cells at each concentration, the concentration interval being approximately 1%.

The dilution error

The cell population technique is carried out by adding a small drop of cell suspension to a drop of protein solution. If the cell suspension is free from extraneous fluid, which is approximately the case if packed centrifuged cells are used, the refractive index of the protein drop remains unaffected. When a considerable amount of liquid is present, however, care has to be taken to ensure that the concentration of protein is unaltered. In general, since cells are suspended in aqueous media of low solid content, the error is one of dilution. It can be calculated in the following way:

Let V = volume of protein drop,

P = % concentration of protein in drop,

v = volume of cell suspension added,

l = proportion of liquid in cell suspension,

i.e. lv = volume of liquid in added drop of cell suspension,

p = equivalent % protein concentration in liquid of cell suspension
(i.e. refractive index of liquid medium is taken as refractive index of water + αp).

After mixing, volume of liquid in the new drop = $V + lv$. Equivalent amount of protein in new drop = $PV/100 + lpv/100$ g. Hence, % protein concentration in new drop

$$P' = \frac{PV + lpv}{V + lv}$$

$$= \frac{P + lp \frac{v}{V}}{1 + l \frac{v}{V}}.$$

Since in practice v is made small compared with V and lv is still smaller, we can ignore higher powers of lv/V and write

$$P' = \left(P + lp \frac{v}{V} \right) \left(1 - l \frac{v}{V} \right)$$

$$= P - l \frac{v}{V} (P - p).$$

Thus the true protein concentration P' is less than the measured one by the amount $(P - p)lv/V$. This error will be greatest when (a) p approaches zero, i.e. the suspension medium is pure water, (b) l approaches unity, i.e. the added suspension is very dilute, and (c) the volume of suspension added is comparable with that of the protein drop. We cannot always control factors (a) and (b) but we can usually make v small compared with V . The maximum error occurs when $p = 0$ and $l = 1$, and is equal to $P(v/V)$. The greatest value of

v/V which can be tolerated then depends on how we wish to regard the error. If we require to work to a relative accuracy of, say 1%, then we must make $v/V = 1/100$. If, on the other hand, we can tolerate an *absolute* error of 0.5% of protein concentration (which in view of inherent biological variation and other factors is probably accurate enough for most work), then for a cell for which $P = 20$ we can have $v/V = 1/40$. In haematological work we can take $P = 35\%$, $p = 8\%$, and $l = 0.5$. Thus for an absolute error of 0.5% we must have $v/V = 1/27$, a condition which is easily met.

By far the simplest method of avoiding the dilution error is to use a centrifuged or sedimented cell suspension for which l may be very small indeed. Such a packed suspension will contain a very large number of cells so that only a small volume is required. Thus the two beneficial conditions of small l and v/V go hand in hand in this case, and the dilution error becomes negligible. If for any reason concentration of the suspension is not possible or inconvenient, the volume v should be kept as small as possible. In practice a very minute drop can be added from a glass pipette drawn out to capillary dimensions over a small flame.

INTERPRETATION OF NUMERICAL RESULTS

Concentration of total solids

As already discussed in Part I, the mean refraction increment for the protoplasm of most cells can be taken as approximately 0.0018. The concentration of total solids is thus obtained by subtracting the refractive index of water from the refractive index of the protein solution in which the cytoplasm becomes invisible and dividing the result by 0.0018. Thus

$$C = \frac{n_0 - n_w}{0.0018},$$

where C is the concentration of solids expressed in grams per 100 ml of protoplasm.

The correction necessary to convert this to grams per 100 g of protoplasm will be dealt with below.

Concentration of water

If the density of the dissolved solids were equal to that of water the concentration of the latter would be simply $(100 - C)$ g per 100 ml. In fact, however, the volume occupied by 1 g of protein in solution (i.e. the specific volume) is 0.75 ml. Hence C g of protein occupy $0.75C$ ml in solution. The concentration of water in a protein solution is thus $(100 - 0.75C)$ g per 100 ml. In the case of protoplasm the specific volumes of the non-protein constituents have to be considered. Lipids, for example, have low densities and high specific volumes (about 1.08). Sugars have a specific volume of about 0.64. Numerous estimates ranging from 0.47 to 0.66 have been given for the specific volume of desoxyribose nucleic acid (see Jungner, 1950). Bearing in

mind the fact that proteins, lipoproteins, and complexes of proteins and carbohydrates form the major solid constituent of most cells, it is unlikely that the specific volume of protoplasm will deviate greatly from 0.75, and in the absence of other information we may assume that the water concentration in a living cell is given with sufficient accuracy by (100-0.75C) g per 100 ml. The error due to the uncertainty in the true value of specific volume is usually small, particularly when C is not great. Thus, taking $C = 16\%$ which is about the value found for many types of tissue cells, and assuming a specific volume of 0.75, the water content would be 88%. Even with a specific volume as low as 0.6, the water content would be 90.4%. At the other extreme, if we admit the very improbable specific volume of unity, the water content would be 84%. Even the extreme range from 84 to 90.4% is not very wide, so that the assessed value of 88% is unlikely to be much in error.

Total wet mass and density

Since 100 ml of protoplasm contain C g of solids and $(100-VC)$ g of water (where V is the specific volume of the dissolved solids)

$$\begin{aligned}\text{total wet mass} &= (100-VC)+C \\ &= 100+(1-V)C \text{ g per 100 ml.}\end{aligned}$$

Hence the density of protoplasm = wet mass per ml

$$= 1 + \frac{1-V}{100}C.$$

If we take $V = 0.75$ as suggested above,

$$\text{total wet mass} = 100 + 0.25C$$

and

$$\text{density} = 1 + C/400.$$

These formulae will be discussed later in relation to experimental work on the density of living cells in Part IV of this series.

Concentrations expressed in terms of wet weight

So far we have expressed the concentration of solids and of water in terms of the *volume* of protoplasm. In other methods of analysis it is more usual to express the results as a percentage of the wet weight. Since the density = $1 + C/400$, the concentration of solids in grams per 100 g of protoplasm

$$\begin{aligned}&= \frac{C}{1 + \frac{C}{400}} \\ &= C - \frac{C^2}{400} \text{ approx.}\end{aligned}$$

In general, therefore, the deviation from C is small, amounting to less than 1 g per 100 g of protoplasm for most tissue cells.

The concentration of water expressed as grams per 100 g of protoplasm is $100 - C + C^2/400$. Again, in most cases the last term can be ignored and $(100 - C)$ is a sufficiently good approximation.

OPTICAL PROBLEMS AND INTERPRETATION

Although the practical determination of refractive index by this method is comparatively simple, a thorough appreciation of a number of points concerning the theory and practice of phase-contrast microscopy is essential if certain pitfalls are to be avoided. Unless the user is prepared to devote the necessary effort to understanding these matters, it would be better not to use the method at all. It is not necessary to employ complicated mathematics, but some form of simple vector theory, such as that elaborated by one of us (Barer, 1951, 1952 *a, b*, 1953, 1954 *a*, 1955), is extremely helpful. An excellent insight into the type of appearance to be expected in a given case can be obtained by the use of the simple vector diagram involving purely geometrical methods.

The vector diagram

As suggested by Zernike (1942), the light vibrations coming from points on a perfectly transparent object can be represented as vectors whose ends fall on the circumference of a circle (fig. 2). The radius of this circle can be arbitrarily chosen as unity. The vector OM is taken to represent in amplitude and phase the light vibration which falls on the object plane. After passing through a perfectly transparent object detail, a phase change ϕ is produced in the vibration. The transmitted light is then represented by the vector OP , where $MOP = \phi$. Thus all transparent details will be represented by points such as P , but with different values of ϕ . M represents a detail of zero phase change, i.e. a point on the clear background, or one having the same transmission and optical path length as the background. Partially absorbing details will be represented by points lying *within* the circumference of the circle. In each case the amplitude of the transmitted vibration will be represented by the distance from the point in question from O , and the intensity by the square of that distance. Hence in conventional microscopy the intensity of all transparent details will be $OP^2 = OM^2 = 1$. In other words, all transparent details, no matter what the value of ϕ may be, will have the same intensity and will therefore appear without contrast. On the other hand, partially absorbing objects represented by points such as V will have intensities such as OV^2 which will be less than OM^2 so that they will appear darker than the background, and contrast will occur. The object of phase-contrast or interference contrast is to shift the origin of the vector circle from O to some new point such as O_1 , so that points like P are no longer equidistant from the origin. For a number of reasons most commercial phase-contrast objectives employ phase plates which advance the direct light by one-quarter wavelength or 90° . In the vector diagram this is equivalent to rotating the

vector OM through 90° to O_1M . In addition the phase plate is usually made to absorb some of the direct light. If the intensity of the direct light is reduced to $1/N$ the amplitude of the corresponding vibration is $1/\sqrt{N}$, so that the new origin lies at O_2 , where $O_2M = 1/\sqrt{N}$. The main purpose of this absorption is to increase the sensitivity for weakly refractile objects. To sum up, therefore, the optical action of the ordinary commercial phase-contrast microscope is represented approximately by a vector diagram such

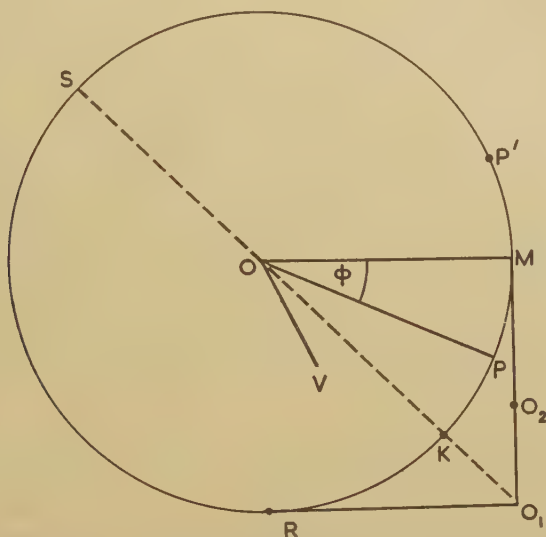


FIG. 2. Vector representation of phase-contrast with a 90° non-absorbing phase plate. For explanation see text.

as fig. 2, in which the origin O_2 lies somewhere on the line O_1M , the exact position being determined by the absorption of the phase plate.

Phase change and intensity

If a homogeneous object of refractive index n_0 and thickness t is immersed in a medium of refractive index n_m , the optical path difference or phase change introduced by the object is defined as

$$\phi = (n_0 - n_m)t.$$

Since in biological work n_0 generally exceeds n_m , the phase change is a delay or retardation, and it is thus convenient to regard retardations as positive. When n_m exceeds n_0 the phase change is an advance, which is regarded as negative. Phase retardations can then be represented by points such as P in fig. 2 and phase advances by points such as P' . This convention is the opposite to that usually employed in mathematics in which angles measured in an anti-clockwise direction are regarded as positive, but it is more convenient in

biological work. It is evident from fig. 2 that a phase advance of ϕ cannot be distinguished from a retardation of $360^\circ - \phi$.

ϕ can be measured either as a unit of length, as a fraction of a wavelength, or in degrees or radians. For reasons stated elsewhere (Barer, 1952 c) measurement in degrees is to be preferred. A phase change of 360° is equivalent to one wavelength.

Non-absorbing phase plates

What one requires to know in practice is how the appearance of an object detail varies with ϕ . This can be readily visualized by considering the vector diagram fig. 2. We shall first deal with the case of a non-absorbing 90° phase plate, for which the new origin is at O_1 . The background intensity will be $O_1M^2 (= 1)$. For a retardation ϕ in the object the intensity will be O_1P^2 . This is less than O_1M^2 , so that the detail appears dark. Maximum darkness will occur when $\phi = 45^\circ$, i.e. for a detail represented by the point K . For points on the circle between K and R the intensity will increase, reaching unity at R , when $\phi = 90^\circ$. For still higher phase changes the intensity will increase above that of the background so that such highly refractile details will appear bright. Maximum brightness will occur at S , when $\phi = 225^\circ$. Finally, the brightness will fall to unity as the phase change approaches 360° at M .

These changes can be calculated and recorded graphically as in fig. 3 (Barer, 1952 a). With this particular type of phase plate ($N = 1$), therefore, dark contrast only occurs with refractile details which introduce a phase change ϕ of less than 90° , i.e. one-quarter wavelength. For phase changes exceeding 90° there will be a reversal of contrast, which for the purpose of this work we designate *false reversal*, as is explained below. Fig. 3 demonstrates other points which are of fundamental importance in interpreting phase-contrast images.

(1) The relationship between intensity and phase change is not even approximately linear except for very small values of ϕ .

(2) There are *two* values of ϕ corresponding to every single value of intensity. This inherent ambiguity is not sufficiently realized by many biologists, who often appear to be under the impression that if one detail is darker than another it must necessarily be more refractile. In fact the very reverse may be the case. For example, in fig. 2 a detail for which $\phi = 45^\circ$ will be maximally dark whereas one for which $\phi = 90^\circ$ will be invisible! This behaviour is an inherent property of all types of phase-contrast and interference-contrast systems. No accurate inferences about refractivity can therefore be drawn from the general appearance of a specimen viewed under one fixed set of conditions alone.

The effects of absorption in the phase plate

Fig. 4 is a vector diagram corresponding to the case of a 90° absorbing phase plate. The new origin is now at O_2 where $O_2M = 1/\sqrt{N}$. Maximum

darkness now occurs at F , a point which corresponds to a phase change of MOF or $\tan^{-1} 1/\sqrt{N}$. This is clearly less than 45° . Similarly reversal of contrast occurs for phase changes exceeding MOF or $2 \tan^{-1} 1/\sqrt{N}$. The critical phase change is now less than 90° . The heavier the absorption in the phase plate, the greater the value of N and the closer will O_2 approach to M .

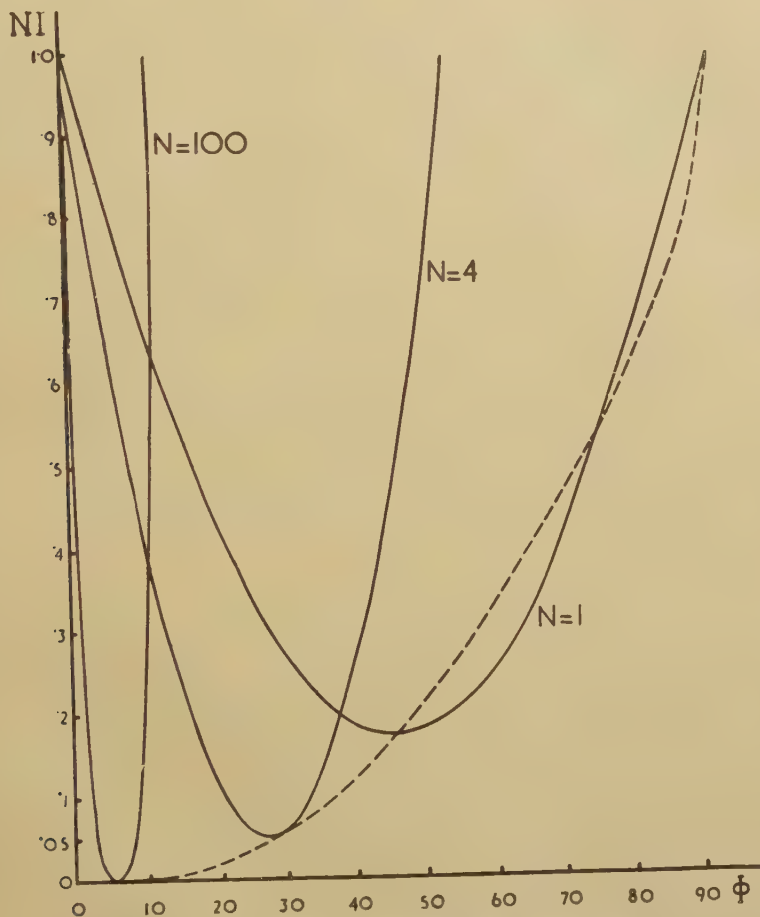


FIG. 3. Relationship between intensity (ordinates) and phase change (abscissae) for 90° positive phase-contrast with phase plates of different absorption. $N = 1$ corresponds to a non-absorbing phase plate, $N = 4$ corresponds to a 75% absorption, and $N = 100$ corresponds to 99% absorption. Note that in each case there are two values of phase change corresponding to a given intensity.

At the same time the maximum darkness and the onset of reversal of contrast will occur with lower and lower phase changes. Finally, when all the direct light is absorbed ($N = \infty$), O_2 will coincide with M_2 and central dark ground illumination in which *all* details appear brighter than the background occurs. The curves marked $N = 4$ and $N = 100$ in fig. 3 show the relationships

between intensity and ϕ for absorbing phase plates, and further illustrate the points discussed above.

Another important effect of having absorption in the phase plate is to increase the sensitivity for weakly refractile details (represented by points on the circle close to M). It can be shown that the sensitivity is proportional to \sqrt{N} . This is reflected in the greater steepness of the intensity curves for $N = 4$ and $N = 100$ in fig. 3.

The point to be stressed, therefore, is that it is almost useless to attempt to compare phase-contrast images unless the absorption of the phase plate is specified. One observer using an objective with very weak absorption may see an object in which the most refractile details appear very dark and in which the weakly refractile details may be virtually invisible. Another observer using a heavily absorbing objective may describe the same object as showing reversal of contrast in the strongly refractile regions while the weakly refractile details may appear dark grey or almost black. Perhaps the chief reason why greater confusion has not so far arisen in the literature is that most commercial phase plates have absorptions lying between 50% ($N = 2$) and 75% ($N = 4$), and over this range the critical phase changes do not vary very rapidly with N . Thus for the 50% phase plate maximum darkness occurs for $\phi = \tan^{-1} 1/\sqrt{2} = 35^\circ 18'$ and reversal for phase changes exceeding $70^\circ 36'$. For the 75% plate the corresponding values are $26^\circ 36'$ and $53^\circ 12'$. With the increasing use of more heavily absorbing phase plates, which are necessary for the highest sensitivity, however, more confusion is likely to arise.

The effect of varying the refractive index of the mounting medium

As stated above, the phase change produced by an object is given by $\phi = (n_0 - n_m)t$. In fig. 4 let us consider the point P which represents an object detail with phase change ϕ (MOP). Now suppose we increase n_m , the refractive index of the mounting medium, very slightly; ϕ decreases slightly so that the same detail will now be represented by a point P' lying a little closer to M . In the particular case shown the detail will therefore appear slightly less dark than before because O_2P' will be greater than O_2P . As n_m increases, P' moves farther along the circle towards M . Finally, when $n_m = n_0$, ϕ will be zero and P' will coincide with M , so that the detail vanishes. This may be called the 'match-point'. Now suppose n_m increases to a value exceeding n_0 ; ϕ now becomes negative so that P' moves beyond M to a point such as Q' . Since O_2Q' is greater than O_2M , the detail will now appear *brighter* than the background. In other words, reversal of contrast occurs, so that in order to determine the refractive index it is necessary to vary n_m until the object becomes progressively lighter, disappears at the match-point, and finally becomes brighter than the background. This procedure may seem simple, as indeed it is in most cases which occur in practice, but there exist two other types of behaviour which may cause difficulty unless their nature is understood. Consider, for example, a detail represented by P_1 between F and L . Such a detail will appear less dark than the one represented by F . On

increasing n_m , P_1 will move towards M . The first thing that happens, therefore, is that the detail becomes *darker* than before, then progressively lighter until the match-point is reached. The fact that increasing the refractive index of the mounting medium may sometimes make a detail appear darker may seem strange, but it is a natural consequence of the properties of phase-contrast systems. The behaviour of a detail represented by a point such as P_2 beyond L is sometimes even more confusing. Such a detail will appear initially with reversed (i.e. bright) contrast. It becomes progressively less bright at first as n_m increases, then as P_2 reaches L it will actually disappear. This is in fact a *false* match-point, at first sight indistinguishable from the

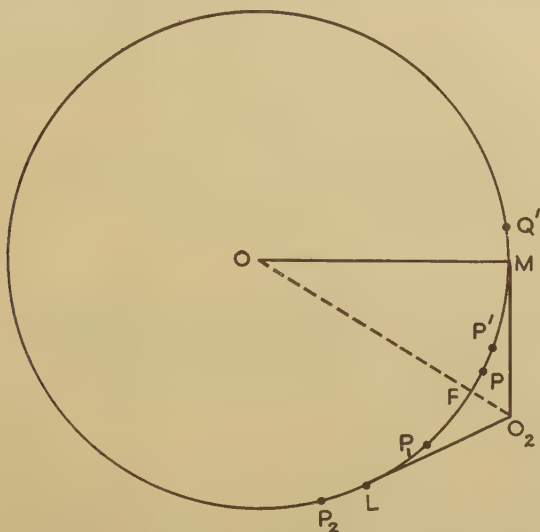


FIG. 4. Vector representation of phase-contrast with a 90° absorbing phase plate. For explanation see text.

true match-point, which occurs when P_2 reaches M . The vector diagram shows how these two cases can be distinguished. Near the true match-point the detail will become bright (i.e. reversed) when n_m is increased. Near the false match-point it will become dark as n_m is increased. It is extremely important to bear this type of error constantly in mind. Since it can only occur with objects showing a relatively high phase change, one should always examine the object first in a medium of the lowest possible refractive index (in the case of living cells, in a saline medium having a refractive index very near 1.334). If the object appears dark in this medium, false reversal is very unlikely to occur in any other medium. If, however, it appears bright, two match-points corresponding to L and M may be expected. The value of the refractive index at the false match-point L tells us nothing of any real value; it can only be used to calculate the absorption of the phase plate. The true match-point is not affected by the characteristics of the phase plate. This does not, however, mean that all types of phase plates are equally suitable.

Sensitivity

In the neighbourhood of the true match-point the phase change produced by an object detail becomes very small. The ability to see detail then depends on the inherent sensitivity of the phase plate, and the accuracy of the refractive index match also depends in turn on the phase plate. It can be shown that for a 90° phase plate, when ϕ is small, the contrast γ with which a detail is seen is given approximately by $\gamma = 2\sqrt{N} \cdot \phi$ (Barer, 1952 *a*). If we assume that a contrast of 10% ($\gamma = 0.1$) is necessary for comfortable discrimination of detail, the smallest value of ϕ which can be observed is $1/20\sqrt{N}$. On considering the least sensitive case of a non-absorbing phase plate ($N = 1$) we see that $\phi = 1/20$ radian $= \lambda/125$ approximately, where λ is the wavelength of the light used. Thus,

$$(n_0 - n_m)t = \frac{\lambda}{125}$$

so that the least detectable refractive index difference is

$$(n_0 - n_m) = \frac{\lambda}{125t}.$$

It should be noted that this quantity depends on the object thickness t , so that the minimum detectable refractive index difference is not an absolute quantity, but depends on the object. For an object of thickness one wavelength (0.5μ)

$$(n_0 - n_m) = \frac{1}{125} = 0.008.$$

This is equivalent to a difference in protein concentration of 4.6%. When $t = 20\lambda$ (10μ), however, the accuracy of matching increases twentyfold so that refractive index differences of 0.0004 or concentration differences of 0.23% can be detected. These figures are actually very conservative. In the first place a contrast of 10% is adequate for *comfortable* visibility; object details can still be seen with very much lower contrast values under fairly good conditions. It is not unreasonable to expect a five- or even tenfold improvement in sensitivity for this reason alone. Secondly, non-absorbing phase plates are rarely used in practice. Since the sensitivity is proportional to \sqrt{N} , for a 75% absorbing phase plate ($N = 4$), the minimum detectable refractive index or concentration difference will be half those given above. Altogether therefore something like a tenfold improvement may be expected in practice. Thus even with objects only 0.5μ thick it should be possible to estimate the solid concentration to within $\pm 0.5\%$. In principle, the sensitivity can be increased without limit by increasing the absorption of the phase plate. Thus a 99% absorbing phase plate ($N = 100$) should be ten times as sensitive as a non-absorbing one. In practice, however, the absorption of the phase plate cannot be increased indefinitely as this is accompanied by increased light-scattering in the objective, which causes glare and limits

contrast. Scattered light can be reduced, but not entirely eliminated, by blooming the objective lens surfaces. There is no doubt that a heavily absorbing phase plate should be used for the present technique. The degree of contrast obtained with many objects near the match-point is extremely low and it is recommended that absorptions of not less than 75% should be used. For best results bloomed objectives with absorptions between 80 and 90% are preferable.

Errors in the refractometry of internal details

It is important to decide how far the technique can be applied to the determination of the refractive index of internal details such as chromosomes, mitochondria, &c., which are themselves embedded in cytoplasm and are therefore not accessible to the surrounding medium. The special difficulties which arise in using ordinary phase-contrast systems for this type of work will be considered later. It will simplify matters to deal first with an ideal case.

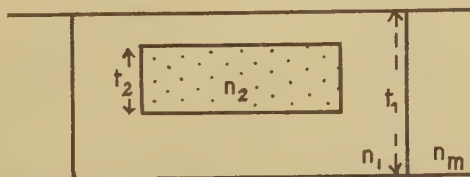


FIG. 5. Diagram representing a granule or inclusion of refractive index n_2 and thickness t_2 embedded in cytoplasm of refractive index n_1 and thickness t_1 , the whole being surrounded by a medium of refractive index n_m .

Fig. 5 represents an object of thickness t_1 and refractive index n_1 , embedded in a medium of refractive index n_m and containing a uniform granule of thickness t_2 and refractive index n_2 . The optical path through any region is the sum of the products of refractive index and thickness. Thus in the region of the granule the optical path $= n_2 t_2 + n_1(t_1 - t_2)$. In the region of the medium, the optical path $= n_m t_1$. Hence the optical path difference or phase difference

$$\phi = n_2 t_2 + n_1(t_1 - t_2) - n_m t_1.$$

At the apparent match-point we must have $\phi = 0$ to make the granule appear with the same intensity as the background. Hence

$$n_2 = n_1 + (n_m - n_1) \frac{t_1}{t_2}.$$

Thus n_2 , the refractive index of the internal granule, can be determined only if both n_1 and the ratio t_1/t_2 are known. n_1 can be measured, but the determination of t_1/t_2 may be extremely difficult except in special cases. It is clear, therefore, that the refractive index of the medium at the apparent match-point of an internal detail bears no simple relationship to the refractive index of that detail. Only in the special case, where $t_2 = t_1$, i.e. when the 'granule' occupies the whole thickness of the cell, is it possible to equate its refractive index to that of the external medium.

We can use the formula derived above to obtain some estimate of the amount of error to be expected when t_2 is less than t_1 . Let us assume that $n_1 = 1.360$ and that at the apparent match-point for the granule $n_m = 1.380$. Suppose first that $t_2 = 9/10 t_1$; then $n_2 = 1.382$. In this case, therefore, the error in assuming that the refractive index of the medium is that of the granule at the match-point is only 0.002 or roughly 1% solid concentration. This is perhaps not very serious. Now suppose $t_2 = \frac{1}{2}t_1$. In this case $n_2 = 1.40$. The error is now 0.02 or about 10% solid concentration. This is far too great to be ignored. Finally, for a very thin granule for which $t_2 = 1/10 t_1$, $n_2 = 1.560$, an error of 0.18. Obviously extreme caution must be used in applying the method to internal details. Unless the thickness ratio is known or can be estimated with reasonable accuracy, it is probably better not to attempt to derive more than semi-quantitative conclusions from such measurements.

Complications due to the halo effect

In the above discussion a somewhat simplified view of the functioning of phase-contrast has been taken. We have in fact regarded practical phase-contrast as an ideal method of interference microscopy and have assumed that it is possible to separate completely the direct from the diffracted components and to treat them differently. In practice, however, this is never possible because some part of the diffracted light must inevitably fall on that area of the phase plate which is occupied by the image of the direct light. Complete separation of the two components is therefore not possible. The degree of overlap can be made less by using a narrow phase ring, but the latter can never become extremely narrow without causing a severe loss of intensity. The use of a phase ring of finite width manifests itself in two ways which are really different aspects of the same effect. First, object details which are made to appear dark are surrounded by a bright halo (and conversely), and second, the true phase-contrast effect is only seen over a limited 'zone of action', that is to say phase-contrast is most marked at edges and discontinuities and falls off rapidly towards the interior of a uniform homogeneous object. Thus, for example, a uniform flat sheet of plastic or mica viewed by positive phase-contrast might appear dark near the edges but would become progressively lighter towards the interior. If the sheet were large enough, the intensity at its centre would approximate to that of the background. Effects such as these can be seen in many published papers. (See, for example, Wolter, 1950.) Very striking practical examples have been given by Saylor, Brice, and Zernike (1950) in their paper on colour phase-contrast. In this method a coloured phase-contrast effect is superimposed on a background of different colour. In some of their photographs the colour at the centre of a uniform object, such as a crystal, is the same as that of the background, whereas the edges show a different colour.

It is difficult to give a rigorous definition of the term 'zone of action'. All one can say is that in the types of phase-contrast microscope used in

practice the conversion of a phase change into an intensity change, which is, of course, the basic aim of the method, is carried out most effectively at the edge of a uniform transparent object. The efficiency of this conversion becomes progressively less towards the interior of the object until eventually there may come a stage at which phase-contrast virtually ceases to operate altogether. The width of the zone of action, which may perhaps be taken as the distance over which phase-contrast operates at all, depends mainly on the

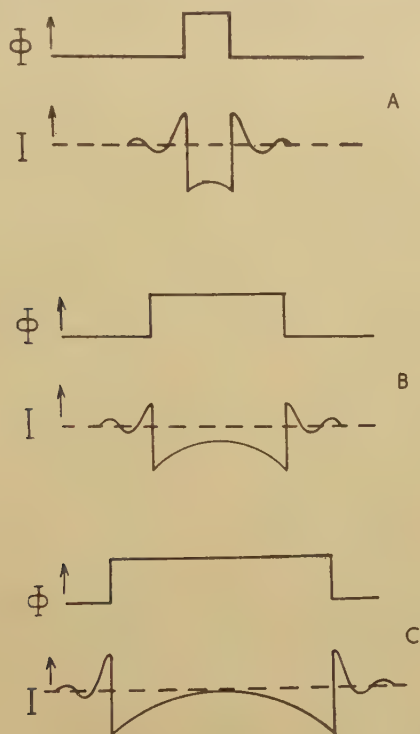


FIG. 6. Diagram showing the intensity relationship in the image of a uniform transparent step as viewed by phase-contrast. For explanation see text.

degree of overlap between the direct and diffracted beams and thus on the ratio of the radial width of the phase annulus to the radius of the entire phase plate. Some very approximate figures which give an idea of the order of magnitude of the zone of action will be given below. The extent of the zone of action does not depend on the degree of absorption of light in the phase annulus, though of course the halo and the accentuation of edge contrast become more marked. The radius of the phase annulus (as distinct from its radial width) affects resolution slightly, but does not seem to have much effect on the zone of action. Some interesting photographs which illustrate these points are shown on pages 134-7 of the book by Bennett and others (1951).

The diagrams in fig. 6 will help to give some idea of the sort of effect which the existence of a zone of action may have on the appearance of various types

of objects. Each pair of diagrams represents a plot of the phase change across the field of view. The objects chosen are thus simple, uniform, transparent steps. Below each step is shown the type of light intensity distribution to be expected in practical phase-contrast. The dotted line represents the background intensity. Around the edges of each step there occurs a bright halo which is in turn surrounded by a number of alternating dark and light fringes. These are frequently quite evident in phase-contrast photographs of some types of objects. The light distribution within the area of the step depends

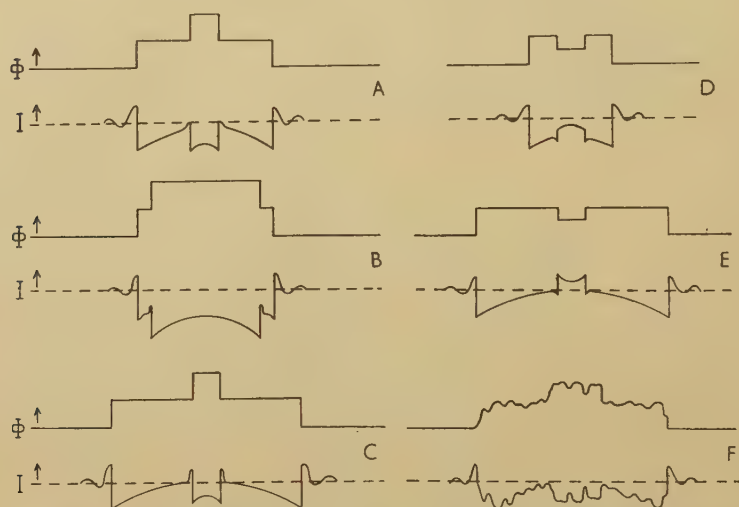


FIG. 7. Diagram showing intensity relationships in the images of compound transparent steps as viewed by phase-contrast. For explanation see text.

on the width of the latter. With very narrow steps the intensity curve resembles the phase-change curve. As the step broadens, however, its interior appears less dark. When the step is sufficiently broad, the intensity at its centre may fall to approximately that of the background as in *C*. Thus the centre of a large uniform area is always at the 'match-point' as compared with the surrounding medium, and changing the refractive index of the latter will have little effect on the appearance of this region.

Fig. 7 shows a number of cases which represent objects with internal details embedded in regions of uniform phase change. Such objects would approximate, for example, to nuclei embedded in cytoplasm, though of course they are greatly simplified. In *A* the 'nucleus' is relatively narrow and at some distance from the edge of the surrounding 'cytoplasm'. Apart from a light halo immediately surrounding it, the edge of the nucleus will appear about as dark as the edge of the cytoplasm, but the centre will be somewhat paler. In *B* the nucleus is broad and surrounded by only a narrow ring of cytoplasm. The cytoplasm and edge of the nucleus will be represented fairly faithfully,

but the nucleus becomes progressively paler towards its centre, until it attains approximately the same intensity as the cytoplasm. In *c* the nucleus is narrow and surrounded by a very broad zone of cytoplasm. In the image the nucleus will look as if it were embedded in a region having the same intensity as the background. In such cases therefore the nucleus is represented as if its phase change were measured relative to the surrounding cytoplasm instead of relative to the embedding medium. *D* and *E* represent cases of nuclei whose optical thicknesses are *less* than that of the surrounding cytoplasm. In *D* the nucleus is shown as appearing paler than the cytoplasm, but nevertheless darker than the medium. In *E*, in which the zone of cytoplasm is much broader, the nucleus would appear reversed irrespective of the refractive index of the mounting medium.

These diagrams are greatly simplified and only furnish a very rough guide to the interpretation of cases which may occur in practice. In particular we have ignored the basic fact that the degree of darkening in positive phase-contrast is not a simple function of phase change, so that regions of greater phase change may sometimes actually appear lighter instead of darker. On the other hand, biological objects are rarely as structureless as those depicted in the diagrams. The more correct plot of the phase changes which occur across a cell might perhaps be something like that shown in *F*. In such a case the falling off of phase-contrast due to the 'zone of action' effect would probably be much less marked. However, the general conclusion that the contrast of the nucleus depends at least as much on the refractive index of the cytoplasm as on that of the background remains valid. If the nucleus is narrow relatively to the cytoplasm, its appearance will be almost entirely dependent on the properties of the latter. If the nucleus is only surrounded by a narrow ring of cytoplasm, its appearance will depend on some sort of average of the properties of the medium and the cytoplasm. A rough estimate of the extent of the zone of action can be made from a knowledge of the width of the phase annulus relative to that of the objective aperture. The halo and zone of action effects can be regarded as due essentially to the presence of a blurred image of the object, of reversed contrast, formed by an objective having the numerical aperture equivalent to the radial width of the phase annulus. If we take the latter as approximately $1/10$ of the radius of the full objective aperture, the zone of action will extend over a distance ten times the resolving power of the objective. With a 4 mm objective of N.A. 0.65, for example, the resolving power can be taken as approximately 0.5μ , so that the zone of action would extend over a distance of about 5μ . In other words, a circular cell of diameter 10μ could be expected to be represented with reasonable accuracy. Small cells such as bacteria should be practically free from the zone of action effect. If the width of the phase strip could be reduced to about $1/250$ of the radius of the objective aperture, the full phase-contrast effect would extend over areas about 250μ in diameter, i.e. about the size of the field of view of the 4 mm objective. Not only would such a narrow phase strip admit very little light, but accurate alignment of the image of the

sub-stage annulus on the phase strip would be very difficult and critical. Any increase in radial width of the phase annulus will inevitably reduce the region over which phase-contrast operates. This will make the halo narrower and more obvious. The system becomes progressively less sensitive to slow variations in phase change as the strip broadens, and eventually only edges or sharp discontinuities exhibit strong contrast. It seems therefore that in practical phase-contrast the intensity is a function not merely of the phase change alone but of the gradient of phase change as well.

These effects are not restricted to ordinary phase-contrast, but occur in all 'imperfect' forms of interference microscopy, i.e. those in which the reference beam and the object beam are incompletely separated. As a rule the degree of overlap of these beams is much less in interference microscopy than in phase-contrast, so that the halo and zone of action are broader, and the halo less conspicuous. They usually only become obvious when the system is adjusted to approximately dark-ground conditions. Even with a 'perfect' interference contrast system the analysis on page 441 shows that although the intensity representation of phase changes may be accurate, nevertheless the refractive index of an internal detail cannot be determined with certainty unless its thickness is known.

One special case may be mentioned, as it frequently occurs in practice. At the match-point of the cytoplasm (i.e. when the latter is embedded in a medium of its own refractive index) the appearance of its inclusions will depend on the product of their thickness and the refractive index difference between the inclusion and the cytoplasm. Thus, in general, inclusions which appear dark at the match-point of the cytoplasm will have refractive indices greater than that of the cytoplasm and conversely, unless the phase change due to the inclusion alone is sufficient to produce false reversal with a particular phase plate.

Finally, since the phase change ϕ at any point is the product of refractive index difference and thickness, local variations in ϕ (and hence in intensity) may be due to variations in refractive index, variations in thickness, or both. At the match-point, however, the whole of a body of uniform refractive index will vanish, independently of any variations in thickness. In these circumstances any areas which remain visible must have refractive indices different from that of their surroundings. Thus the immersion method can be used to distinguish between local variations in thickness and variations in refractive index or solid concentration.

Our thanks are again due to the Rockefeller Foundation and the Royal Society for making this work possible and to the Medical Research Council for providing a personal grant to S. J.

Fig. 3 is reproduced by kind permission of the Editors of the *Journal of the Royal Microscopical Society*.

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The Cell-theory: a Restatement, History, and Critique

Part V. The Multiplication of Nuclei

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SUMMARY

1. The belief that nuclei arose by *exogeny*, without relation to pre-existent nuclei, was due mostly to Schleiden (1838). Kölliker (1843) supposed that new nuclei arose by *endogeny* within pre-existent nuclei.

2. Other early theories of the origin of nuclei contained a considerable element of truth. Many early workers thought that the ordinary nuclei of many-celled plants and animals multiplied by *division* (Bagge, 1841; Nägeli, 1844; von Baer, 1846), or by the *disappearance* of the old nucleus and its immediate *replacement* by two new ones (Nägeli, 1841; Reichert, 1846).

3. The history of the discovery of *mitosis* falls into three parts.

In the first (1842-70), chromosomes were seen accidentally from time to time, but no special attention was paid to them (? Nägeli, 1842; Reichert, 1847).

In the second (1871-8), metaphases and anaphases were repeatedly seen, placed in their right sequence, and recognized as normal stages in nuclear multiplication (Russow, 1872; Schneider, 1873; Bütschli, 1875; Strasburger, 1875).

In the third (1878 onwards), the main features of prophase and telophase were described and it was shown that the chromosomes replicated themselves by longitudinal division (Flemming, 1878-82). The separateness of the chromosomes in prophase and the constancy of their number were discovered (Rabl, 1885).

4. These researches proved that in ordinary mitosis the nucleus neither disappears completely nor divides. In certain Protozoa, mitotic division of the nucleus is a reality.

5. The indirect origin of cells, through the intermediacy of syncytia, was established by the work of Nägeli (1844), Rathke (1844), Kölliker (1844), and Leuckart (1858).

6. There is nearly always a cellular phase at some stage or other of the life-history of organisms, even when all the somatic tissues are syncytial. Certain Zygomycetes provide an exception.

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INTRODUCTION

WE are still concerned with Proposition III in the formulation of the cell-theory adopted in this series of papers: that is to say, with the proposition that *cells always arise, directly or indirectly, from pre-existent cells, usually by binary fission*. In Part IV of the series (1953) we traced the history of the discovery that cells multiply by division. As we saw in Part II (1949), cells are defined by their possession of *protoplasm* and a *nucleus*. In cell-division the protoplasm divides (Part IV, 1953). It remains to trace here the history of our knowledge of the way in which nuclei multiply. We shall concern ourselves with the discovery that nuclei are genetically related to pre-existent nuclei, and with the gradual revelation of the real nature of that relationship. The history of the discovery of the rest of the process of mitosis (the behaviour of the centrioles, asters, &c.) is irrelevant to our purpose and will not be considered. The remainder of the paper will be concerned with the indirect origin of cells from cells, by the production of syncytia and the subsequent formation of cells in or from these.

The discussion of Proposition III will be completed in Part VI of the series, which will deal with the continuity of cells from generation to generation.

A few words about the purpose of this series of papers would, I believe, be appreciated by some readers.

The cell-theory has been subjected to powerful attack. As a result, its validity has been questioned in zoological textbooks. I decided to study the evidence against it very carefully, in the original papers. Having done this, and examined the whole subject more widely, I reached the conclusion that the theory withstood the attacks. I then decided to try to persuade others of its validity. I found that I could only develop my argument and make myself understood by a historical treatment, with critical comments from the modern point of view. In many fields of science we must recognize an embryology of ideas: our modern outlook can only be fully grasped and assessed if we understand the causes that make us think as we do. This applies particularly to the cell-theory. Though I have great respect for the history of science, yet my main purpose in this series of papers has not been to write history, but to use a mainly historical method to establish what I believe to be an important truth about living organisms.

EARLY THEORIES OF NUCLEAR MULTIPLICATION

Before telling the history of the discovery of cell-division, it was necessary, in Part IV of this series of papers, to describe the wholly erroneous theories that were for long entertained about the process by which cells multiply. Our understanding of the multiplication of nuclei has come in a different way. Some of the early theories were wrong, but they were not wholly wrong; and considerable interest attaches to them in so far as they led towards the discovery of mitosis. First, however, it is necessary to eliminate a theory that con-

tained no element of truth. This was the theory that nuclei arise exogenously in what Schwann (1839, pp. 45 and 207-12) called a *Cytoblastem*, without any relation to pre-existing nuclei.

Exogeny

Valentin (1835, p. 194) appears to have been the first person to make a suggestion as to the origin of nuclei. He claimed that in the chorioid coat of the eye, nuclei arise by a process of precipitation. He confuses his remarks by calling the nuclei *Pigmentbläschen*, though they are colourless; the globules of pigment appear subsequently round them.

The theory that nuclei in general arise exogenously was due to Schleiden (1838, pp. 145-6). His ideas have already been given in detail in Part IV of this series of papers (1953, p. 416), and need only be briefly mentioned here. It may be remembered that in his view, a nucleolus appeared without any relation to a pre-existing nucleus, and the nucleus or *Cytoblast* was formed round this by deposition of a granular coagulum. This nucleus then produced a cell round itself. In his first paper (1838*a*), Schwann accepted Schleiden's scheme and applied it to animals. It was unfortunate that the first ideas about the multiplication of nuclei were completely wrong, yet supported by two famous investigators.

Henle (1841, pp. 153-4) was evidently affected by these beliefs. He shows a cartilage-cell (his plate V, fig. 6) with a nucleus containing a nucleolus at one end and a body resembling a nucleolus at the other. He suggests rather tentatively that a nucleus had just formed round one of the nucleoli. Kölliker at one time thought that nucleoli might appear spontaneously in certain cases, by the crystallization of granules in a homogeneous fluid, and that nuclei were subsequently formed round them (1844, pp. 143-4 and 150); but, as we shall see (p. 452), he supposed that nucleoli ordinarily multiplied by division within nuclei.

Nägeli at one time allowed that nuclei might originate without any relation to pre-existent nuclei (1846, see especially pp. 62-63).

During the eighteen-forties, the belief that new nuclei arose in some sort of connexion with pre-existing ones became quite general, but the older view still lingered on. One cannot fail to regret that Remak, who had done so much to elucidate the multiplication of cells, eventually retracted a little from the position he had taken up and began to equivocate. He came to believe that new nuclei might in certain cases originate independently of pre-existing ones. He thought that when small blood-vessels were developing in the cutis of the frog, new nuclei appeared that were not related to the embryonic nuclei; he remarked also that the stellate cells (presumably fibroblasts) of connective tissue developed without any known connexion with the cells of the embryo. He also thought that new cells originated in diseased tissues without any participation of pre-existing nuclei (1862, p. 282).

Remak was not the only distinguished investigator to continue to hold such views. Weismann (1863*a*), in his account of the development of the egg of

Chironomus, says that nuclei 'appear' (*erscheinen*) at the same moment over the whole of the blastoderm, which then separates itself off round each of them and thus forms uninucleate cells. Lankester (1875, pp. 38-41) thought that in the development of *Loligo*, the 'autoplasts' (nuclei of the yolk-epithelium) were of the same nature as the nuclei of the blastomeres, but for the most part of independent origin. (The actual origin of the yolk-epithelium of cephalopods was finally revealed in the next decade by Vialleton (1888).)

Endogeny

Kölliker appears to have been the only person who claimed that new nuclei arise endogenously within old. Mainly as a result of his studies of the embryology of nematodes and of the frog, he reached the conclusion that the nucleolus lengthens, constricts, and divides; a new nucleus then forms endogenously round each of the two nucleoli thus produced, within the membrane of the mother-nucleus (Kölliker, 1843; 1844, pp. 143-4, 150). All this he described in the puzzling nomenclature that has already been described (Part IV, p. 418). Later he summed up his opinion in very clear language. 'Nuclei and cells multiply by endogenous procreation', he wrote; 'nucleoli by division' (1845, p. 96).

The theory of endogeny bore little relation to the actual events of nuclear multiplication, but at least it involved a genetic relationship between old and new nuclei. The two theories to be discussed next came nearer to reality. Indeed, each of them revealed a considerable part of the truth.

Division

Even at the present day we often read in biological textbooks of nuclear division, though in fact, of course, typical nuclei do not, in any intelligible sense, divide. Exceptions to this are provided by certain Protozoa (see below, p. 474), and also by the polyenergid nuclei of certain other members of the same phylum (see Baker, 1948a); but the latter are better regarded as representing aggregations of many small nuclei. It is true that the nuclei of certain tissue-cells of higher animals have been supposed to multiply by 'amitosis'; but in fact it seems unlikely that such a rough-and-ready method could divide the gene-complex accurately enough to produce viable cells (though it might suffice in a syncytium). It is more likely that in these cases disguised mitoses occur, without regular, easily recognizable metaphases and anaphases.

Ehrenberg (1838) appears to have been the first to witness the multiplication of a typical nucleus. He saw clearly the nucleus of the protomonad flagellate *Monas vivipara*, but regarded it as the testis. He remarks (pp. 9-10) that it divides when the animal divides, and he gives a figure (his plate I, fig. IVa) professing to show a stage in this process.

Barry (1841, *a*, *b*, and *c*) thought that nuclei multiply by division, or rather fragmentation, and that the fragments become new *cells*. This view was accepted by Goodsir (1845, p. 2). Barry worked chiefly with the red blood-corpuscles of various vertebrates. His observations on this subject, however,

are so unsatisfactory that they cannot be regarded as having contributed to knowledge. In an earlier paper (1839) there are some passages (on p. 361) that suggest at first glance that he witnessed nuclear multiplication in early mammalian embryos, but this is not so.

Bagge (1841) reported nuclear division in the early embryo of '*Ascaris nigrovenosa*' (= *Rhabdias bufonis*). He recognized that the duplication of the nucleus preceded that of the cell. His terminology is unfortunately misleading: he calls nuclei *cellulae* and cells *vitelli partes* or *globulae*. His illustration, here reproduced as fig. 1, is probably the earliest attempt to represent consecutive stages in the process of nuclear multiplication.

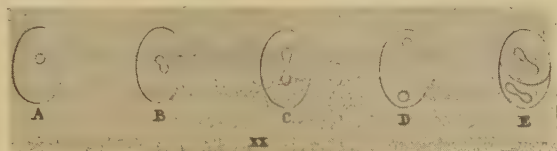


FIG. 1. Stages in supposed nuclear division in *Rhabdias bufonis*. Bagge, 1841 (fig. xx).

Remak (1841) studied blood-formation in the late chick-embryo. He noted that in dividing blood-cells the two nuclei were joined together by a stalk-like process (probably the remains of the spindle). Later (1845) he tried to follow the way in which nuclei multiply in the developing striated muscle of the frog tadpole. He was at first hesitant, remarking cautiously, 'I am not able to assert that the formation of new nuclei proceeds always from those already present, though several observations suggest this.' Further study of the same object convinced him that the new nuclei arise by division of the old (1855, p. 154), and he illustrates what he takes to be a division stage (his plate XI, fig. 5). Neither the text nor the figure gives any details of the process. Writing about nuclear multiplication in general, he admits (p. 174) that the process has not been elucidated with certainty, but claims that it clearly begins with a constriction. He leaves it undecided whether the nuclear membrane dissolves.

Valentin briefly described and figured a stage in the division or *Spaltung* of the nucleus of a cell in the membrane covering the auricles of the frog's heart (1842, p. 629 and plate VII, fig. 95 *bis*, *a* and *b*).

Breuer (1844, p. 31) and his associate, Günsburg (1848, pp. 361-2), claimed that in regenerating mammalian skin, nuclei multiplied by division or fragmentation (*sejunctione*). These authors may perhaps have been looking at the nuclei of polymorphs. Günsburg thought that the nucleus generally fell into as many pieces as there were nucleoli.

Nägeli (1844) described the division of the nucleus of a germinating spore of *Padina* (Phaeophyceae). His figure showing the two nuclei, supposed to have been produced by division of the old nucleus before the cell had divided, was reproduced on p. 433 of Part IV of this series of papers (1953). Nägeli subsequently came to regard division as the usual method of nuclear multiplication in plants (1846, pp. 68-69), though not, as we shall see (p. 457), the only method.

With von Baer we enter a new phase. His account of the process of nuclear multiplication was far fuller and more accurate than anything that had been published previously.

Perhaps because he published (in German) in a Russian journal, perhaps because his paper takes the shape of an informal, chatty letter from the seaside, von Baer's contribution (1846) to our understanding of the multiplication of nuclei has not received the credit it deserves. During his stay at Trieste he artificially fertilized the eggs of '*Echinus*' (*Paracentrotus*) *lividus* and watched the process of cleavage. In his description we can follow what was actually happening. It is very helpful to place beside his description a set of figures of the cleavage of the same animal made much later by Hertwig (1876). These figures are here reproduced in fig. 2, as an illustration of von Baer's paper. They show what can be seen in life. It will be noticed that Hertwig did not see the chromosomes (though he saw them clearly enough when he fixed and stained the embryo). Von Baer's great merit is that he gave a realistic description of what can be seen of mitosis when the chromosomes themselves are not seen.

Von Baer correctly identifies the nucleus (*Kern*) of the unfertilized egg. He tells us that on fertilization it sinks more deeply into the egg, and its limits become more difficult to see. He does not recognize the participation of the nucleus of the spermatozoon in the process, but thinks that the egg nucleus alone is the progenitor of those of the embryo. 'After some period of rest', he writes, 'this nucleus, up till now spherical, lengthens rather quickly by sprouting, as it were, at both sides; both ends swell, but the middle becomes thinner and soon divides completely, so that two comet-shaped nuclei with their tails lie opposite one another. Then, very quickly, the tail-shaped appendages pull themselves back into the spherical or vesicular masses, and one has two nuclei. . . . Before the division the original nucleus had already increased in volume; during the division this happens still more, so that each of the two new nuclei has apparently the size of the original one.' The egg now divides. 'Soon afterwards each of the two nuclei now begins to sprout out in the same way, and, dividing in the middle, changes into two new nuclei, round which the yolk-mass then likewise divides, and the whole egg resolves itself into four masses adhering to one another. . . . Quite similarly there follows the division of the quadrants, and indeed in such a way that the direction of the new sprouts stands at right angles to the immediately preceding ones. So it goes on with new divisions, for a nucleus forms itself in advance for each portion of the yolk by division of one that was produced earlier.' He remarks here that a pellicle is formed round the nucleus each time after a period of rest. 'Up to the division into 32 yolk-bodies, when the process is occurring quite regularly, I have been able to watch the division continuously.' The appearance is now for a time less clear. 'But still, when the embryo has left the egg-membranes and is moving itself by means of cilia, each granule or histogenetic element (vulgarly called a "cell") has a very evident nucleus, and they all appear to be derived from the original nucleus of the egg.' Movement of the larva now

Fig. 15.

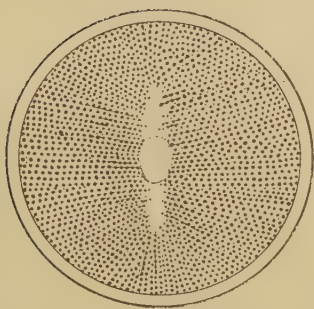


Fig. 16.

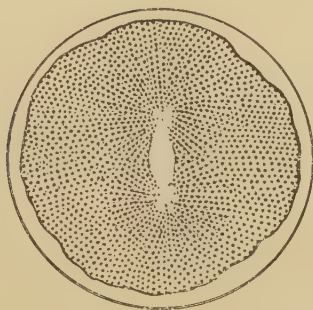


Fig. 17.

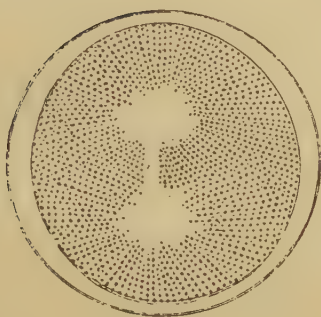


Fig. 18.

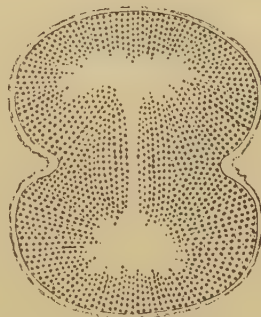


Fig. 19.

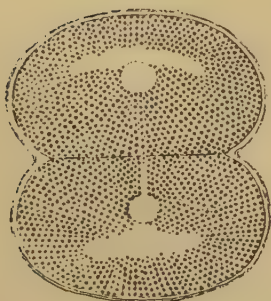


Fig. 20.

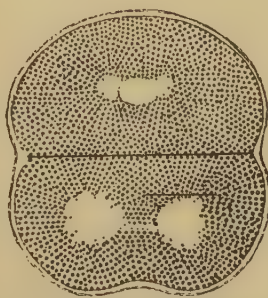


FIG. 2. Stages in nuclear replication in the embryo of *Paracentrotus lividus*, to illustrate von Baer's description. Hertwig, 1876 (plate XII).

makes observation difficult. 'But I have reason for the belief that the permanent tissue-constituents also arise from the original one by quite similar divisions. According to this, the divisions of the yolk would only be the beginnings of the histogenetic separation that progresses continuously up to the final formation of the animal' (1846, cols. 237-40).

Von Baer obviously saw the spindle and regarded it as a nucleus that had elongated in preparation for division. This outlook has not quite left us even today. One finds in textbooks statements to the effect that the spindle is formed from the nuclear sap. In fact, the nuclear membrane disappears during late prophase in most organisms other than certain Protozoa, and the nuclear sap then merges indistinguishably with the ground cytoplasm. Even if one disregards the fact that part of the spindle is often clearly formed in the cytoplasm while the nuclear membrane is still intact, it is still unjustifiable to derive the spindle exclusively from the nucleus. Even if this were not so, it would still be wrong to speak of nuclear multiplication by division; for in those cases in which the spindle-remnant survives to be divided across at cell-division, the products of its division are not incorporated in the new nuclei.

Virchow (1857) considered that nuclei ordinarily divide by a process of constriction. Gegenbaur (1858, pp. 9-10) saw something of nuclear multiplication in the cleavage of the egg of *Sagitta*. He admits that the details of the process escaped him, but he remarks that he saw a stage in which the nucleus was drawn out to a great length, and many were provided with constrictions. He presumably saw spindles and took them for elongated nuclei. He supposed that the actual division of the nucleus must take place very quickly.

Schultze (1861, p. 11) followed up his famous definition of a cell (see Part III of this series of papers (1952), p. 165) with a generalization on nuclear multiplication in the very next sentence. 'The nucleus and also the protoplasm', he wrote, 'are division-products of the same components of another cell.'

In his study of the development of *Musca vomitoria*, Weismann (1863*b*, p. 162) announced that each of the four pole-cells (primary germ-cells) divides into two, with simultaneous division of their nuclei.

As we shall see, a number of botanists had adopted the view that the nucleus disappears at cell-division and is somehow replaced by two new ones. Hanstein (1870) devoted a paper to the refutation of this belief. He worked chiefly with the parenchyma of various flowering plants. He satisfied himself that the nucleus did not disappear. He claimed that it was constricted by a delicate but optically perceptible halving-boundary (*Halbirungsgrenze*), and that when this process was complete, the two halves of the nucleus moved apart to opposite poles and a new cell-wall was formed between them (pp. 230-1).

Disappearance and replacement

The supporters of the theory of nuclear division performed a useful service by calling attention to the fact that two new nuclei are somehow derived from

one old one, but they overlooked a rather obvious part of the usual process—the disappearance of the nuclear membrane and nucleolus. While some investigators were claiming that nuclei divided, others insisted that on the contrary a nucleus *disappears* and is *replaced* by two new ones. Each side in the controversy had seized upon one aspect of the truth.

If a nucleus completely disappeared and was then replaced by two new ones, the latter could be regarded as having arisen exogenously; but it seems desirable to draw a distinction between the origin of a new nucleus without any relation to a pre-existent nucleus, and the disappearance of one nucleus and its replacement by two new ones.

From his studies of pollen-formation, Nägeli (1841) concluded that when the mother-cell is about to divide, the cytoblast (nucleus) is absorbed. A new cytoblast then appears in each of two granular areas in the cytoplasm. Membranes form in such a way as to enclose each of the granular areas. The whole process is then repeated, with disappearance and replacement of the cytoblast. Thus four cells are formed, each with its nucleus. Alternatively, the four cells with their nuclei may be formed simultaneously after the disappearance of the nucleus of the mother-cell. Later, in a general account of pollen- and spore-formation, Nägeli repeats this general scheme, with the added complication that the original nucleus of the mother-cell, lying against the cell-wall, disappears and is replaced by a central nucleus, which in turn disappears and is replaced by four new ones or by two which are each subsequently replaced by two (1844, pp. 83–84 (p. 84 is accidentally numbered 48)).

As we have already seen (p. 453), Nägeli regarded division as the usual method by which the nuclei of plants multiply; but he retained his belief that in particular cases there is absorption and replacement (1846, p. 70).

Hofmeister thought it certain that the nucleus of the pollen mother-cell of *Tradescantia* underwent dissolution (*Auflösung*) and replacement by two new nuclei (1848b, col. 651).

Meanwhile, similar results were being obtained with animals. Reichert studied the egg and embryo of the nematode *Strongylus auricularis* (1846, pp. 201 and 255–6). He described the disappearance or *Hinschwinden* of the germinal vesicle and the mixture of its contents with the substance of the rest of the egg. A new nucleus was formed, but this again disappeared. A new one was formed in each of the first two blastomeres; these again disappeared before the next division. So the process went on. The nucleus underwent *Hinschwinden* before each division, the newly-formed cells contained no nucleus, and finally a new nucleus appeared in each. Reichert followed the repetition of this process up to the stage at which the form of the little worm had become visible. He illustrated his findings by careful drawings (his plate IX). Similarly, Krohn described the disappearance of the nucleus at each cleavage division in the ascidian *Phallusia*, and the reappearance of a nucleus in each newly-formed blastomere (1852, pp. 314–15).

Much later than this, at a time when chromosomes had often been seen, it was still supposed that the germinal vesicle of the primary oocyte did in fact

wholly disappear when the polar bodies were about to be given off. This is perhaps not surprising in view of the large size of the vesicle in relation to that of the chromosomes. Thus van Beneden described the complete disappearance of the germinal vesicle in the rabbit (1875, p. 692). He remarks that at this stage the egg is what Haeckel called a *Cytode*; that is to say, a lump of cytoplasm not containing a nucleus (see Haeckel, 1866, pp. 273-4).

Auerbach also used the term *Cytode* for the cell in which the nucleus has disappeared before cell-division (1876, p. 258). He thought that the substance of the nucleus intermingled with the cytoplasm and dissolved in it. For this reason he termed the stage of mitosis at which the nucleus becomes no longer visible *die karyolytische Figur* (p. 222).

MITOSIS

It is a fact that there is genetic continuity between old nuclei and new, but nuclei do not ordinarily multiply by division. It is a fact that the nuclear membrane and nucleolus disappear at cell-division, yet the whole of the nucleus does not vanish. The truth could only be established when the erroneous parts of each theory had been eliminated, and when the remainder had been integrated by the discovery of something important that had been overlooked by both—the chromosomes.

If we study the old papers in which the early descriptions of chromosomes appear, it seems at first almost impossible to give an intelligible exposition of the way in which our modern knowledge was achieved. Yet there is an evolutionary story to be told, for there were in fact *stages* in the history, more evident, no doubt, to us who look back than to those who lived through the events.

In the first stage there were mere accidental records of bodies that we can now recognize as chromosomes. In the next, metaphases and anaphases were repeatedly described, and came to be regarded as usual stages in the process of nuclear duplication. It was understood that the anaphase was subsequent to the metaphase. In the third and last stage, the prophase and telophase were carefully described, and the real nature of the genetic relationship between one nucleus and the two that succeed it was disclosed through the genius of Flemming.

The first period (1842-70)

In a general account of the changes of form of the nucleus, Henle (1841, pp. 193-4) makes some remarks that suggest strongly that he saw some of the stages of mitosis. He says that nuclei often become oval and then more elongated, and then change into thin striations. The nucleoli disappear and the nucleus then becomes decomposed into a row of little dots (*Pünktchen*). He mentions that nuclei are sometimes connected by threads. If he had left it at this, we should probably have believed that the striations represented the spindle and the *Pünktchen* the chromosomes; but he illustrates what he saw

by drawings, and a study of these suggests that he was not looking at stages in cell-division after all.

Valentin (1842, pp. 630-1) says that after treatment with acids, nuclei are sometimes seen in the act of division, 'with granular accessory appendage'. One can only guess whether the granules were chromosomes. No details are given that could guide us.

Nägeli is the first person of whom we can say that he probably saw chromosomes. In his account of the formation of pollen in *Lilium tigrinum*, he describes how the cytoblast of the mother-cell vanishes and is replaced by a variable number of small cytoblasts, which are transitory and in their turn disappear (1842, pp. 11-12). He figures a cell containing seven of them (see

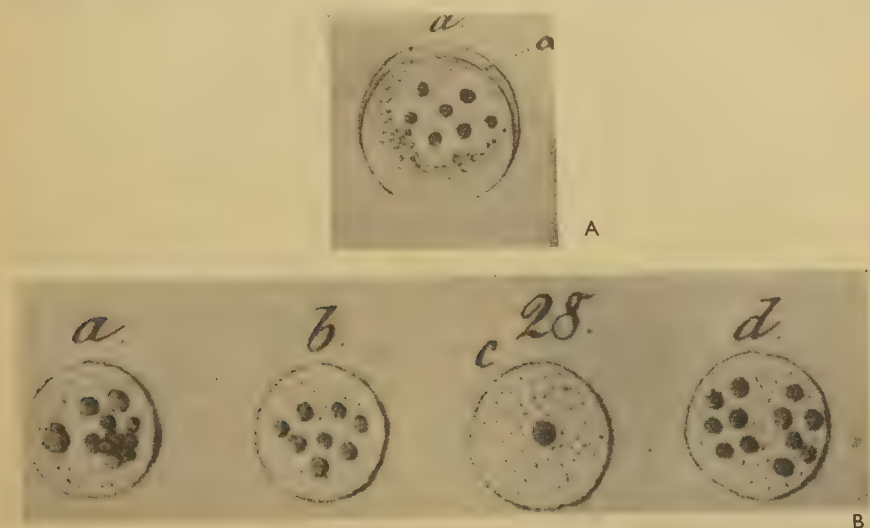


FIG. 3. These are probably the earliest illustrations of chromosomes. A, 'transitory cytoblasts' in the pollen mother-cell of *Lilium tigrinum*. B, the same, in pollen mother-cells of *Tradescantia*. Nägeli, 1842 (plates I, fig. 12d, and II, fig. 28; enlarged from the original figures).

fig. 3, A). When they have disappeared, the contents of the cell divide in two, and a new cytoblast is formed in each of the products of division. It is possible that these transitory cytoblasts were chromosomes, though doubt is engendered by the fact that in some cases there were only one or two of them in the cell. He saw similar appearances in the pollen mother-cell of *Tradescantia* (see fig. 3, B). It seems likely in this case that the bodies were actually chromosomes. Three of the cells illustrated contain 11, 8, and 12 such bodies. Nägeli thought it probable that when these transitory bodies had disappeared, a single, large cytoblast was formed (in the case of *Tradescantia*), and this divided in two (p. 13).

Later, in describing what was presumably the division of the pollen mother-cell of *Amaryllis*, he gives a figure of what he calls the *Kern* but is actually the spindle (1844, plate II, fig. 24a). He remarks that this body has 'dunkle

körnige Anhänge'. These may have been anaphase chromosome-groups, but the figure is unfortunately on too small a scale to show them.

If Nägeli probably saw chromosomes, then Reichert (1847) certainly did so. He investigated carefully the spermatogenesis of *Strongylus auricularis*, noting that the developmental stages could be followed in time-sequence by passing along the testis from its blind end. After mentioning what were evidently the spermatogonia at the blind end of the tube, he goes on, 'Besides these, vesicles [cells] sometimes occur which contain two nuclei, of exactly the same microscopical constitution, but smaller [than those of the other cells], and also vesicles that are provided with no nuclei at all, but only with separate small granules (*Körnchen*); these vesicles cannot be mistaken for the clear vesicles that are perhaps of artificial origin' (p. 101). Serious artifact is indeed unlikely, for Reichert simply opened the testis at chosen places to let out the contents, and wetted the preparation with saliva or egg-white (p. 99). The *Körnchen* were evidently chromosomes, probably those of spermatogonial mitoses, for he thought that the nucleus actually disappeared altogether in what we should call the meiotic divisions (pp. 110-13).

Hofmeister (1848a) had the great merit of realizing that most contemporary cytologists were devoting a disproportionate amount of attention to the cell-wall and neglecting the nucleus. He decided to study nuclear phenomena in the pollen mother-cells of *Tradescantia virginica*. He found that as the cell grew, so did its nucleus and nucleolus; but eventually, at about the same time, the nucleolus and nuclear membrane disappeared. As we have already seen (p. 457), Hofmeister believed in the actual *Auflösung* of the nucleus in *Tradescantia*, and its replacement by two new ones. He thought, however, that when the nucleus had just dissolved, the albuminous material occupying what had been its site was in a particularly coagulable condition. He produced coagulation by the action of either water or tincture of iodine. Either of these agents produced separate *Klumpen* in the cell (cols. 427-30). These objects, which he supposed to be artificial coagulates, were in fact chromosomes (see fig. 4). He considered them to be of the same nature as the transitory cytoblasts of Nägeli.

Nineteen years later Hofmeister still retained the same opinion. As a result of studies of the formation of pollen in various phanerogams and of spores in vascular cryptogams, he concluded that at the stage of disappearance of the nuclear membrane, the substance of the nucleus is easily coagulated as a little clot of strongly refractive substance, or else in the form of numerous, much smaller objects (1867, p. 81). The latter were undoubtedly chromosomes. He



FIG. 4. The chromosomes in a pollen mother-cell of *Tradescantia virginica*, after treatment with tincture of iodine.

Hofmeister, 1848 (plate IV, fig. 10b).

mentions that in spore-formation in *Equisetum* they are situated in the equator of the cell; in *Psilotum* they arrange themselves in a horizontal plate. Fig. 5 is a reproduction of Hofmeister's illustration of a meiotic metaphase in *Psilotum 'triquetum'* (= *nudum*). Fifty-four bodies, presumably chromosome-pairs, can be counted in this figure. The haploid chromosome number in this species is probably in fact 52 (see Manton, 1950, p. 239). Hofmeister's figure shows a remarkable resemblance to the same stage in *P. flaccidum*, as illustrated by Manton (her fig. 236; 52-54 chromosome-pairs). One cannot fail to be struck by such an accurate chromosome-count at this early date.



FIG. 5. The chromosomes in a spore mother-cell of *Psilotum nudum*. Hofmeister, 1867 (fig. 16, e).

Hofmeister once again attributed the appearance of chromosomes to artificial coagulation by water. In the circumstances it is surprising that he should have given such an accurate representation of their number.

Virchow (1857, p. 90) saw a cell with what he calls a branched nucleus among dividing cells in a cancerous lymph-gland. His figure suggests that he may possibly have seen a metaphase (his plate I, fig. 14).

Henle (1866) saw what were apparently pachytene stages (his figs. 263, 268) and perhaps the metaphase and telophase of the first maturation division (fig. 266) in mammalian spermatogenesis. His figures and descriptions are too vague, however, for certainty to be reached. He used acetic and chromic acids as fixatives (pp. 355-6), and one would expect the meiotic chromosomes to have been visible. Indeed, it is rather strange that there are so few early accounts of chromosomes in testes, for it would only have been necessary to examine the organs of almost any animal during the season of spermatogenesis to see at any rate the chromosomes of the first prophase. Such stages were seen much later by Spengel (1876) in the spermatogenesis of several genera of Gymnophiona. He compared them to Chinese writing (p. 31, see his plate II, fig. 33).

Chromosomes were probably seen by Krause (1870) in the epithelial cells of the surface of the cornea of various mammals. He called them granulated oval corpuscles (*Körperchen*). He thought that the nuclei of the epithelium multiplied by division, but did not claim that the corpuscles were necessarily connected with this process. Subsequently he gave an illustration of these bodies (1876, fig. 8, f). The figure seems to represent a late prophase.

The first period of chromosome studies, which started with Nägeli in 1842, ended with Krause's paper of 1870. Up to the latter date the descriptions and figures were vague and unsatisfactory, so that we generally cannot tell exactly what stage of mitosis or meiosis was seen, and we cannot even be certain, in some cases, that chromosomes were seen at all. No attempt had been made as yet to arrange the stages of mitosis in a time-series.

The second period (1871-8)

During the second period discoveries about chromosomes came with a rush. Kowalevski (1871) published the first figure of chromosomes that really

resembles the object. One can tell instantly on looking at his drawing that he saw an anaphase (see fig. 6). His object was a section through the embryo of the lumbriculid worm '*Euaxes*' (= *Rhynchelmis*), at the moment when the first set of micromeres was being given off. He used chromic acid for hardening the egg. He calls the two groups of chromosomes *zwei körnige Anhäufungen* (p. 13). He regarded them as representing division-products of the nucleolus.

Russow (1872) carried our knowledge of chromosomes much further in a study of spore-formation in vascular cryptogams. He found that at the division of the spore mother-cell, a *Stäbchenplatte* was formed (pp. 89, 126). He saw these metaphase plates particularly clearly in ferns and Equisetales. Leaving for a moment the cryptogams that form the main subject of his very long

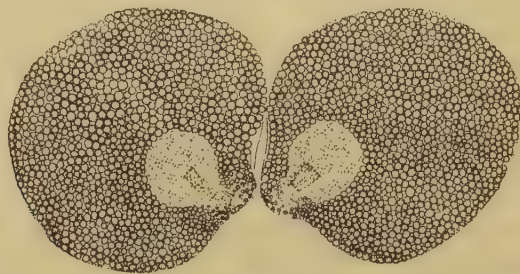


FIG. 6. Anaphase at the formation of the first quartette of micromeres in *Rhynchelmis*. Kowalevski, 1871 (plate IV, fig. 24).

paper, he remarks that he has seen these plates most clearly of all in the pollen mother-cells of *Lilium bulbiferum*. He then proceeds to the first serious attempt ever made at a description of chromosomes. He remarks (p. 90) that in *Lilium* they are short, worm-shaped corpuscles or slightly curved rodlets, colourless, pale, and faintly refractive, scarcely detectably stained by iodine, and almost instantaneously dissolved by alkalis (even at great dilution) and by ammonium carminate; also by chlor-zinc-iodide, without becoming coloured. He noted the same chemical behaviour in the chromosomes of the vascular cryptogams.

He distinguished clearly between the *Stäbchenplatte* and the subsequently-formed *Körnerplatte* (cell-plate).

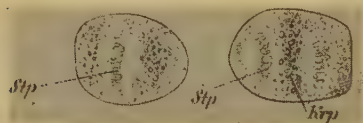


FIG. 7. Metaphase and anaphase in the spore mother-cell of *Ophioglossum vulgatum*. Russow, 1872 (plate VI, figs. 121 and 122).

Russow also saw anaphase chromosome-groups (see fig. 7). He called each of them a secondary *Stäbchenplatte*. He did not explain how they arose, and evidently thought they were the metaphase plates of the next division (pp. 127, 204). He noted that when there is a *Stäbchenplatte*, there is never a nucleus, and put forward the possibility that the plate is formed from the nucleus. He noted (p. 90) that when a secondary plate is viewed from the side, it resembles a very granular nucleus. This suggests that he saw a telophase.

Russow strongly denied that the chromosome plate is an artifact, as Hof-

meister had supposed. He saw it in the intact sporangia of *Polypodium* and '*Aspidium*' (= *Dryopteris*).

Schneider's paper of 1873 constitutes a landmark in the history of our knowledge of chromosomes. He followed carefully the cleavage of the egg of the rhabdocoel *Mesostomum ehrenbergii*. By using acetic acid as fixative, he clearly saw the *dicke Stränge* or chromosomes, and noticed that one half of them went to one pole and the other half to the other (see fig. 8). He saw mitosis not only during cleavage, but also during later embryonic stages and in the adult (pp. 113-16 and plate V, fig. 11). He knew that the nucleus had been thought by others to disappear at cell-division, but he considered it probable that the process he had described in *Mesostomum* actually occurred in these cases. It is evident that he thought that amitotic division also took place. Schneider's paper is above all important for its clear seriation of metaphase and anaphase.

Tschistiakoff (1875, *a* and *b*) saw metaphases and illustrated them clearly in spore mother-cells of *Isoetes* (Lycopodinae) and various ferns, and also in pollen mother-cells of *Magnolia* (his plate I, figs. xx and xxiv). He called the chromosome-plate a *Körnchenlamelle* (1875*a*, col. 1). He made the serious mistake of supposing that the two new nuclei were formed at the poles of the spindle while the chromosomes were still on the metaphase plate (1875*b*).

A paper of this period by Ewetsky (1875) is remarkable because it contains the first reasonably good figure of a prophase (see fig. 9). The cell is from the endothelium of Descemet's membrane in the eye, in regeneration following operational damage. Like Russow, Ewetsky called the chromosomes vermiform structures. Like so many others, he thought that the spindle was the nucleus and that it divided, merely enclosing the anaphase chromosome-groups at its ends.

Bütschli (1875) studied polar body formation and cleavage in the nematode *Cucullamus elegans*, a parasite of fresh-water fishes. He saw metaphases and anaphases distinctly. Unfortunately his paper is not illustrated. The drawing shown here (fig. 10) is from his publication of the following year. He gives



FIG. 8. The earliest figure showing stages in mitosis in correct sequence. The first cleavage of the egg of *Mesostomum ehrenbergii*. Schneider, 1873 (plate V, fig. 5).



FIG. 9. An early representation of prophase. An endothelial cell of Descemet's membrane. Ewetsky, 1875 (plate V, fig. 7).

a particularly clear account of the occurrences at the first cleavage (1875, pp. 211–12). He says that the nucleus becomes invisible and a longitudinally striated, spindle-shaped body appears. Each fibre is swollen at the equator of the spindle into a *Korn* or *Körnchen*, and if one looks from the end of the spindle one sees a ring of granules (*Körnchenkreis*). The error of supposing that chromosomes were swollen regions of spindle-fibres was hard to eradicate in subsequent years. It may be remarked that Bütschli's name for the group of chromosomes at metaphase was a much more realistic one than the term

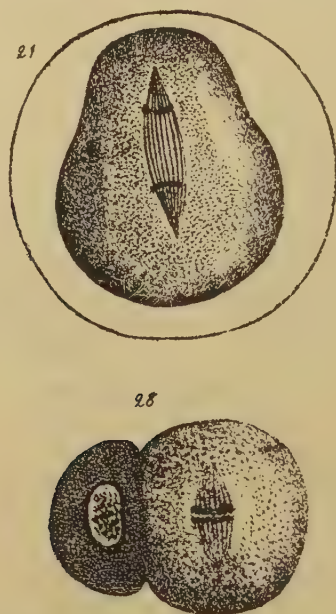


FIG. 10. Typical illustrations of mitosis by Bütschli. Anaphase of the first cleavage and metaphase of the second in *Cucullanus elegans*. Bütschli, 1876 (plate III, figs. 21 and 28).

Platte, which still survives; for there is no real resemblance to a plate unless the chromosomes happen to be very small, very numerous, and very close together. From the single ring of granules now arise two rings, and these move apart. Bütschli uses the expressive term *Auseinanderrücken der Körnchenkreise* for the anaphase. Although he used acetic acid as a fixative in the investigation, it seems possible that he watched the anaphase in life. He noticed that the spindle and chromosomes disappeared and a new nucleus was formed at each pole. He gives no details of these occurrences. He showed the negative merit of not regarding the new nuclei as division-products of the spindle.

Bütschli, like others, had already (1873) seen and figured spindles (but not chromosomes) in the micronuclear mitoses of *Paramecium*, and he recognized the same object in *Cucullanus*. In 1873 he had regarded the spindles as seminal vesicles.

In his paper of 1875 Bütschli described the fusion of chromosomal vesicles to form a nucleus, but he did not relate the vesicles to chromosomes. Such

vesicles had already been reported by Remak (1855, p. 139 and plate IX, fig. 14), Lang (1872), and Oellacher (1872, pp. 410-11 and plate XXXIII, figs. 29-36). These investigations, however, did not help towards the elucidation of mitosis.

Strasburger was drawn into the study of mitosis by his interest in the process of fertilization in conifers. He worked particularly with the spruce, *Picea vulgaris*. The first two mitoses after fertilization escaped him, but he studied the multiplication of the four resulting nuclei, which are situated at the end



FIG. 11. Typical illustrations of mitosis by Strasburger. Metaphase, anaphase, and telophase in the multiplication of the nuclei at the lower end of the ovum of *Picea vulgaris*. Strasburger, 1875 (plate II, figs. 27 and 30).

of the ovum that is farthest from the micropyle. He used material fixed in alcohol, without staining. He saw metaphases and anaphases clearly (see fig. 11, upper figure). His description (1875, pp. 26-27) shows that he thought the process was one of actual nuclear division. He regarded the spindle, with its equatorial *Platte* of *Stäbchen* (chromosomes), as the nucleus. When the *Platte* separated into two, at the beginning of anaphase, the nucleus had divided. Each daughter-nucleus was formed by the fusion of the chromosomes of one plate with one another and with half the spindle. Strasburger made scarcely any attempt at a description of prophase or telophase, though he did illustrate a telophase (fig. 11, lower figure).

Strasburger realized the necessity of studying the process in life, so as to be sure that alcohol did not produce artificial appearances and also so as to be able to place the stages in their proper sequence with certainty. He found suitable material in *Spirogyra*. He followed the whole process of mitosis in the living alga (1875, pp. 33-46 and plate III), though he was not able to see much of prophase or telophase beyond the disappearance and reappearance of the

nucleolus. He also studied alcohol-preparations, and confirmed their reliability. (Several years later (1879) he found a particularly suitable object for vital studies of mitosis in the staminal hairs of *Tradescantia*, immersed in 1% cane-sugar solution.)

Having found close resemblance between the processes of mitosis in such widely different plants, Strasburger now investigated it in many other diverse forms, and saw similar metaphases and anaphases over and over again. The cells he studied all had short chromosomes, and it is probable that this fact prevented a more complete understanding of what really happens in mitosis.

Strasburger was not content to study plants only. He knew of Bütschli's work on *Cucullanus*, and got into touch with him. Bütschli provided him with unpublished figures of mitosis in *Cucullanus* and of meiosis in *Blatta*, and Strasburger recognized the similarity to what he had seen in plants. Strasburger himself studied mitosis in mammalian cartilage (1875, pp. 186-9) and especially in the cleavage of the ascidian, *Phallusia mamillata* (pp. 189-97).

Strasburger brought the whole of his work together in his justly famous book, *Ueber Zellbildung und Zelltheilung* (1875), in which he included (with full acknowledgement) some of Bütschli's unpublished figures. This book was by far the most complete account of cell-division available at the time, and served to show the universality of mitosis as the ordinary process of nuclear multiplication.

At the time, the writings of Bütschli and Strasburger attracted far more attention than those of the Russian cytologists and Schneider. A number of authors were quick to recognize, in their own research-material, the descriptions given by the two former workers. Van Beneden was one of the first to come under their spell (1875). He studied the process of nuclear duplication in the ectoderm of the rabbit embryo, after fixation with osmium tetroxide and staining generally with picrocarmine (a favourite combination with early students of chromosomes). He recognized the separation of the equatorial plate of refringent globules or *bâtonnets* into two *disques nucléaires*, and the movement of these apart from one another at anaphase. He noted correctly that the new nuclei were formed from the disks, which swelled up at the expense of the surrounding cytoplasm and became less and less easily stainable. Later, in the course of the work that resulted in the foundation of the group Mesozoa, van Beneden saw mitosis in the cleavage of the cell that gives rise to the infusiform embryo of *Dicyemella* (1876, pp. 48-52; plate I, fig. 28; plate III, figs. 2, 4, 11).

Mayzel (1875), also influenced by Bütschli and Strasburger, saw various stages of mitosis in the regenerating corneal epithelium of the frog, including a metaphase with radially-arranged chromosomes (p. 851).

The rarity of Strasburger's first edition reflects the publisher's underestimation of the interest of this new line of research. A new edition was quickly produced (1876), with advice from Bütschli in correspondence and conversation. In the same year Bütschli produced an immense paper (1876), profusely

illustrated with figures of mitosis in the cleavage of a leech, of *Cucullanus* (see fig. 10), and of *Limnaea*, of meiosis in the testis of *Blatta*, and of nuclear changes in the conjugation of various ciliates. It is strange to note his pre-occupation with side-views of metaphases and anaphases. Prophases he scarcely noted. He shows one, however, in *Cucullanus* (his plate III, fig. 20) and suggests that it may represent a preliminary stage (p. 226). If only he had studied metaphases more often in polar view, he might have made important discoveries about the constancy of chromosome number. He still describes the equatorial plate as consisting of the thickened parts of the spindle-fibres (p. 219). Telophase still eludes him, but he thinks that new nuclei must arise from the groups of chromosomes at the two ends of the spindle (p. 220).

Balbani (1876) found an excellent source of mitotic figures in the ovariole epithelium of the nymph of the grasshopper *Stenobothrus*. He saw the prophase chromosomes as short, unequal rods and followed them through all the stages of mitosis till they fused at telophase to form a mass that became vacuolated; a membrane then appeared round it. He says that each of the *bâtonnets* cuts itself into two halves, but he gives no indication of the direction of the cutting. This short paper is, for its period, a remarkably complete account of mitosis in a single kind of cell.

The study of mitosis was now taken up actively by O. Hertwig, who produced a succession of papers on polar-body formation and cleavage in leeches, heteropods, echinoderms, and frogs (1876, 1877, 1878, *a* and *b*). An exponent of the osmium / carmine technique (and also of others), his careful studies were marred only by a tendency to follow Bütschli in regarding the chromosomes as swellings of the spindle-fibres. These papers were important because they revealed new facts about polar bodies and fertilization rather than because they established new details of the process of mitosis; but they helped to show how widely applicable were the findings of Bütschli, Strasburger, and the rest.

Eberth (1876) saw mitotic figures in the regenerating cornea of the frog and rabbit, and compared them with Strasburger's descriptions. The latter, in an interesting critique of Eberth's findings, discusses the origin of the spindle (1877, p. 522). He denies that it is derived simply from the nucleus. Sometimes there is no distinction between nuclear sap and cytoplasm at the time when the spindle is formed: the two have become continuous with one another. This message from the past deserves attention at the present day.

One cannot better comprehend the deficiencies of knowledge about mitosis at the close of the second period than by studying the third edition of Strasburger's book, which was published a little later (1880). Though the stages of metaphase and anaphase were by this time so familiar, they were not in the least understood. Strasburger still believed that the *Kernplatte* or equatorial 'plate' of chromosomes became divided at metaphase, and that this division was of a hit-or-miss nature (pp. 331-3). He thought that rod-shaped chromosomes ordinarily arranged themselves along the length of the spindle. Division took place in the same way whether the chromosomes were rods or granules.

If any part of a rod or granule was in the equatorial plane at metaphase, that granule or rod was divided across at that place. Those granules or rods that lay nearer one pole of the spindle passed towards that pole without division. Longitudinal division never occurred except in those particular cases in which long chromosomes arranged themselves at metaphase wholly in the equatorial plane. The splitting then occurred at metaphase.

The third period (1878 onwards)

Five years after the publication of Strasburger's third edition the chief facts of mitosis had been established, chiefly by the brilliant researches of Flemming and Rabl.

Strasburger, as we have seen, had already followed mitosis in the living cells of *Spirogyra*. Particular stages in mitosis had been seen in the living cells of animals. Mayzel (1877), for instance, had put various cells of vertebrates in aqueous humor and seen the stages that he had previously (1875) studied in fixed preparations. No one, however, had watched the actual succession of the stages in animal cells. Now, in 1878, three papers were published by investigators who had seen the process of mitosis unroll before their eyes. Schleicher was the first in the field, with a very short account of mitosis in living cartilage-cells of various Amphibia (8 June). Peremeschko, who had read Schleicher's paper, was next (27 July). He studied epithelial cells, fibroblasts, leucocytes, and endothelial cells of blood-vessels in the tails of young newts ('*Triton*' (*Triturus*) *cristatus*). Schleicher's and Peremeschko's studies were of importance in confirming beyond question the order of the most striking events of mitosis. Peremeschko remarked that the threads were sometimes *knäuelförmig* at the beginning of the process; so evidently he saw something of the prophase. Neither of these authors, however, added any important new facts.

Flemming had already (1877) chosen the salamander (*Salamandra maculata*) as his cytological research-material, on account of the large size of the cells and nuclei in this animal. He now started his research on cell-division, and read a paper on the subject at Kiel on the very day (1 August) on which he first saw a copy of Peremeschko's. This paper (1878) was short, in marked contrast to his massive later contributions. It contains more than just the foreshadowings of the important discoveries that were to come. His research-material was again the salamander, especially its larva. He studied the urinary bladder, the epithelium of the skin and gills, cartilage, connective tissue, the endothelium of blood-vessels, and blood-cells. He followed the whole process of mitosis in life and also studied fixed and stained preparations. Flemming was outspoken in his criticism of Strasburger's scheme of mitosis. Here in this paper one finds the first serious attempt at a description of the *Anfangsphasen* or early prophase-stages. Flemming definitely derives the chromosomes (*Fäden*) from the stainable substance visible in the form of a *Gerüst* in the interphase nucleus. He traces the gradual shortening and thickening of the trabeculae of the *Gerüst* to form finally the chromosomes of the metaphase *Stern*. He considers that the disappearing nucleolus supplies material to the thickening chromo-

somes. He notes that the chromosomes are split longitudinally throughout their length in (late) prophase, a discovery of the first importance, but in this paper he does not trace one longitudinal half to each pole in anaphase. He gives no exact account of what happens at metaphase. He sees the chromosomes move apart at anaphase, and describes the changes of telophase as resembling those of prophase in reverse. Thus for the first time the chromatin was followed through from one resting nucleus to the next. As a result of his studies, Flemming denied that the nucleus could be said without qualification to divide.

Flemming's choice of organisms with long chromosomes as his research-material, both in this early work and later, undoubtedly helped him to elucidate the main features of mitosis.

Flemming now began to produce a succession of papers, the main purpose of which was to show the uniformity of the process of mitosis. He denied that amitosis had been proved to exist in the tissues of animals, except possibly in leucocytes. Indirect nuclear

division, with *Fadenmetamorphosen des Kerns*, was the rule (1879a, pp. 21-22). He now reverted to the *Längsspaltung* of the chromosomes. He saw the longitudinal split in both prophase and metaphase (see fig. 12) and suggested tentatively that one longitudinal half of each thread might go to each daughter-nucleus (1879b, p. 384). In the same paper he describes the prophase. He makes the mistake of supposing that the chromosomes are

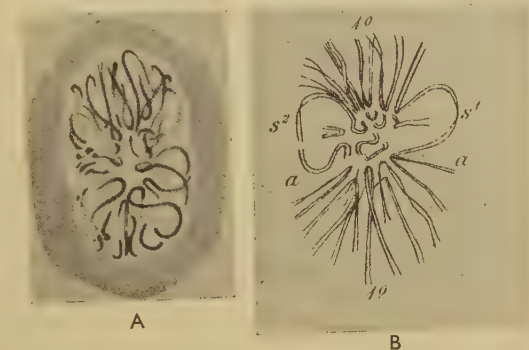


FIG. 12. The earliest figures showing the longitudinal splitting of chromosomes. A, epithelial cell from the gill of a salamander larva, to show the longitudinal split in some of the chromosomes at prophase. B, endothelial cell in a small blood-vessel of a salamander larva, to show the longitudinal split at metaphase. Flemming, 1879b (plate XVII, figs. 7 and 11).

joined end to end into a continuous *Knäuel*. This he describes as a thin thread, which thickens and eventually breaks across into separate chromosomes. He once more describes the telophase as prophase in reverse, and gives a tabular synopsis of the whole process, arranged to bring out this resemblance (p. 392). He follows the telophase *Knäuel* into the network (*Gerüst*) of the interphase nucleus.

Flemming emphasized that longitudinal splitting occurs constantly in diverse kinds of cells (1880, p. 212). He doubted Strasburger's belief that there were considerable differences between one case and another, and turned to plant material to find whether his scheme applied there also. Removing the coverslips from someone else's preparations of the embryo-sack of *Lilium croceum*, he restrained them to his own satisfaction and was able to confirm

that what he had so often observed in animals occurred also in this case (1882a, p. 43). He denied that the chromosomes fuse at metaphase to form a plate.

Flemming's great achievement was his discovery that in mitosis one longi-

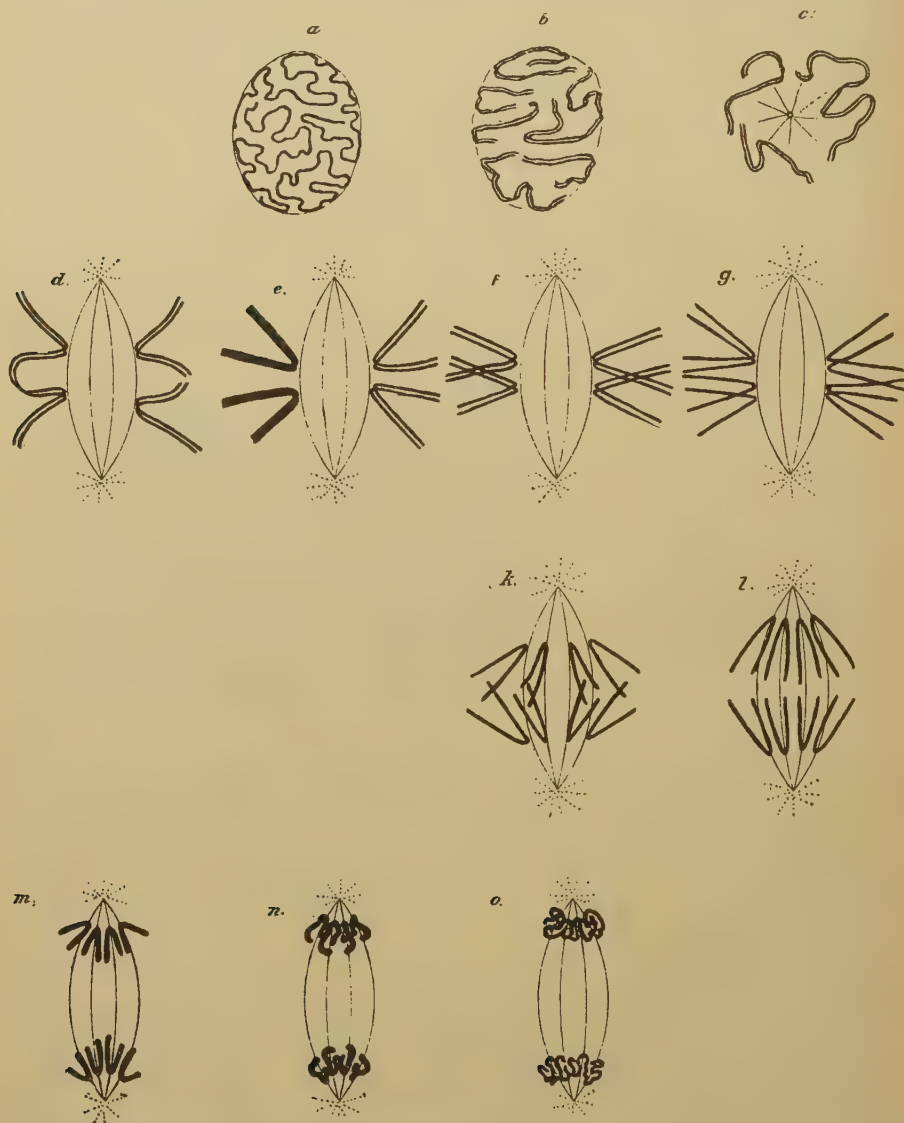


FIG. 13. Diagram of mitosis. Flemming, 1882b (plate VIII, fig. 1). (*h* and *i* are here omitted. Flemming used them to illustrate Strasburger's opinions.)

tudinal half of each chromosome goes in each direction, so that each daughter-nucleus is formed from a complete set of longitudinal halves. He brought together his ideas on the basic plan of mitosis in a diagram published in his book, *Zellsubstanz, Kern und Zelltheilung* (1882b). The diagram is reproduced

here as fig. 13. The only error of any importance is the joining together of the chromosomes in prophase, end to end, to form a continuous *Knäuel*. The achievement represented by this diagram can be appreciated when it is remembered that only a few years earlier, almost nothing was known of prophase or telophase, and the most essential fact of mitosis—the separation of the chromosomes into two groups of longitudinal halves—was quite unknown.

Rabl's work (1885) was complementary to Flemming's, for it corrected the latter's main error and made good his main deficiency. His material was the epidermis of the floor of the mouth and of the gills of the larva of *Salamandra maculata*, and various organs of *Proteus*. He showed that in the prophase, the chromosomes are not joined end-to-end in a continuous *Knäuel*, but are separate from one another from the first. Further, their number is the same as at metaphase, and this number is always the same (24) in various cells of the two species studied. Rabl carried out his extremely laborious work with the utmost care and skill. One of his figures of a prophase, with separate, numbered chromosomes, is shown here in fig. 14. By 1880, as Boveri (1904, p. 4) remarked, the investigations of Flemming, Strasburger, van Beneden, and others were already leading to the conclusion that the number of chromosomes in the cells of each species was the same or nearly the same, but the paper that decided the issue was Rabl's of 1885. Boveri had great admiration for the Austrian cytologist. He writes of his 'wonderful perseverance and observational capacity' (1888, p. 5). Rabl studied carefully the early prophase and late telophase stages, and his findings in this field, taken in conjunction with his establishment of the constancy of chromosome number, formed the basis on which Boveri started to build his theory of the individuality or continuity of the chromosomes.



FIG. 14. 'The chromosomes in a prophase nucleus of an epidermal cell of the larva of *Salamandra maculata*. Twenty-two of the twenty-four chromosomes are shown. Rabl, 1885 (plate XIII, 6th figure).

Nomenclature

It is curious that cytologists should have been so slow to suggest international technical terms for the description of mitosis.

Schleicher pointed out the inconvenience of the multiplicity of terms used for the process itself, and suggested *Karyokinesis* (1879, p. 261). Flemming remarked that even in direct nuclear division, there is movement in or at the nucleus, which is what Schleicher's term means. He therefore suggested the substitution of *Karyomitosis* for it, to indicate 'thread-metamorphosis in the nucleus'. Instead of having to say 'nuclear division figures', one might use the short word *Mitosen* (1882b, p. 376). It must be regretted that Flemming was so far influenced by his profound study of the long chromosomes of the

salamander as to choose this word, for in very many organisms the metaphase chromosomes cannot by any stretch of the imagination be described as threads.

The terms *Prophase*, *Metaphase*, and *Anaphase* were coined by Strasburger (1884, pp. 250 and 260). He did not use the terms metaphase and anaphase exactly as they are used today. For him, the metaphase continued until the daughter-chromosomes were quite separate from one another. Today, the anaphase is usually regarded as starting as soon as the spindle-attachments begin suddenly to move towards the poles. There would be little or no difference between the two usages of the word, however, in the case of the short chromosomes with which Strasburger usually worked. His anaphase included the modern anaphase (or late anaphase) and also telophase. His failure to give a separate name to the latter reflects his lack of attention to the final stage of mitosis.

The word *Telophase* was coined by M. Heidenhain ten years later (1894, p. 524). Curiously enough, he did not define it by any change in the chromosomes. His telophase started directly the centrosomes (*Mikrocentren*) left their positions at the poles of the spindle to migrate to their final sites in the new cells.

Interphase was introduced by Lundegårdh (1913, p. 211) to indicate the period between two closely consecutive mitoses. He drew a distinction between interphase and the *Ruhezustand* that follows mitosis when another division will not occur, or will be indefinitely delayed (pp. 213-19). He described differences between nuclei in interphase and those in the resting state.

Very diverse terms were used for chromosomes before that word was at long last introduced. They were called *Fäden*, *Kernfäden*, *Schleifen*, *Stäbchen*, *stäbchenförmige Körner*, *Körnchen*, and *chromatische Elemente*. Waldeyer certainly performed a useful service when he introduced *Chromosomen* (1888, p. 27). It would have been difficult to choose a shorter word so applicable to the object named in all its variant forms.

Comment

It is a strange fact that some of the best early workers on chromosomes continued to believe that nuclei ordinarily multiplied by division. Auerbach did not fall into this error. He contributed to the subject a short paper that did not receive the attention it deserved. As we have seen (p. 458), he at first believed in the actual solution of the whole of the nucleus at cell-division. Later, when the participation of chromosomes in the process had been repeatedly described, he justly claimed that there had been an element of truth in his belief. He denied that the spindle is derived solely from the nucleus, and that the main mass of it participates in the formation of the new nuclei; and he protested against the statement that nuclei divide (1876).

To resolve fully the question whether mitosis is nuclear division it would have been necessary to know the structure of the interphase nucleus, but even today this is a subject on which we are very imperfectly informed. Very large chromosomes, especially those of certain Liliaceae and Urodela, have attracted

a great deal of attention from students of mitosis, but there is reason to believe that the nuclei to which they give rise are untypical of nuclei as a whole. As E. B. Wilson remarked (1925, p. 82), the commonest type of nucleus in both plants and animals is the vesicular. Now it would appear, from the important studies of Manton (1935), that the chromosomes occupy a relatively small space in a vesicular nucleus. Independent work by various authors on diverse cells suggests that as a rule the chromosomes of a vesicular nucleus are situated just below the nuclear membrane (see, for example, Ludford, 1954). When each of them has a single heterochromatic segment, it is relatively easy to make sure that these parts of the chromosomes at any rate occupy this situation (Manton, 1935). The form and position of the remainder of the chromosome cannot be defined with certainty, but it appears probable that the euchromatic segments are drawn out into threads in the same part of the nucleus. The nuclear sap occupies a large part of the volume of the nucleus, and the nucleolus (often single) is usually large.

There are all varieties of nucleus between the typical vesicular one just described, through the intermediate types investigated by Chayen and others (1953), to the kind of nucleus that results from the telophase transformation of very large chromosomes. This kind has been carefully studied by Manton (1935) in *Allium ursinum*. It appears that in this species the chromosomes maintain their early telophase positions throughout the interphase, simply swelling up to form almost the whole of the nucleus, so that there is very little room for nuclear sap. The nucleoli are not free to move, and therefore remain separate.

Pollister (1952) has very clearly described and figured two contrasting theories of nuclear structure, but it seems probable that what he describes are really the extreme forms of an object that varies widely in different plants and animals.

Of the regular constituents of the nucleus—membrane, sap, chromosomes, and nucleolus—only the chromosomes can be said to divide in ordinary mitosis. Except in certain Protozoa (see p. 474), the nuclear membrane disappears, and it then becomes impossible to distinguish sap from ground cytoplasm. It is therefore wrong to state definitely that the spindle is formed from the nuclear sap. The spindle is in fact in some cases divided by the formation of a cell-plate, or by the ingrowth of a cleavage-furrow; but there is no evidence either that it is of purely nuclear origin, or that any part of it constitutes a part of the daughter-nucleus; so that even when it is divided, it is not a continuously self-reproducing body. In the present state of knowledge it is not possible to say what parts of the cell, beyond the chromosomes, are concerned in the formation of the daughter-nuclei; but so far as is known, there is ordinarily no direct genetic relationship between the old and the new nuclear membrane, and the same applies to the nuclear sap and nucleolus.

It follows that when *Omnis nucleus e nucleo* was written in imitation of *Omnis cellula e cellula*, the similarity of the two phrases tended to hide an essential difference. The word *e* was being used in two different senses. The

ground cytoplasm of a cell arises from that of a pre-existent one by mere division. Two new nuclei ordinarily arise from a pre-existent one by an entirely different process. No new nucleus will be formed unless a group of anaphase chromosomes is present, and since these chromosomes arose *e* the old nucleus, we may say that the new nucleus to that extent arose *e* the old. But the new nuclear membrane, nuclear sap, and nucleolus did not in any intelligible sense arise *e* the old.

The basic truth of the old Latin aphorisms nevertheless remains. Neither a cell nor a nucleus exists, unless there has been a pre-existing cell and nucleus which gave rise to it either directly (in the case of the cytoplasm) or indirectly (in the case of the nucleus). In Part II of this series of papers (1949) the cell was defined by its possession of protoplasm and nucleus. It is a fundamental part of the cell-theory that these are both, directly or indirectly, self-reproducing parts. The only reservations that must be made about this rule are that protoplasm must originally have evolved from matter that did not possess all its qualities, and that the nucleus, as we know it today in the great majority of plants and animals, must have evolved from a simpler structure in the distant past. The question whether it is legitimate to speak of a nucleus in the Cyanophyceae and Bacteria, and whether there is anything in those groups that throws light on the origin of the definitive nucleus, must be reserved for consideration under Proposition VI.

Although mitosis is not in fact a process of nuclear division in the great majority of plants and animals, including those in which it was first studied, yet mitotic division is a reality in certain Protozoa. Indeed, most of the errors about mitosis, entertained by the early workers on the subject, are not errors at all in the case of many flagellates. It was discovered by Blochmann (1894) that in several species of *Euglena* the nuclear membrane never disappears during mitosis, but simply becomes constricted across. Further, the nucleolus elongates into a rod thickened at each end and finally breaks in the middle, leaving one nucleolus in each of the daughter-nuclei. As Blochmann pointed out, the process is nevertheless mitotic, for chromosomes participate in it. Indeed, their behaviour is nearly normal, except that their arrangement at the stage corresponding to metaphase is less regular than usual. Keuten (1895), who had participated with Blochmann in the original work, was able to show that the chromosomes divide longitudinally and that their division-products separate in the usual way. No definite spindle or centrioles are seen. Alexeieff (1911) showed that this form of mitosis, far from being restricted to *Euglena*, occurs also in protomonads and peridinians, and indeed in certain non-flagellate Protozoa.

The more recent work of several authors, especially Hollande (1942, pp. 111-15), has confirmed the general correctness of Blochmann's and Keuten's findings. In the polymastigine, *Tetramitus*, the process is even more similar to ordinary mitosis, for a spindle is formed and the chromosomes arrange themselves very regularly at metaphase; but here again the whole

affair occurs within a persistent nuclear membrane, and the nucleolus duplicates itself by division (Hollande, 1942, pp. 185-7).

Dangeard (1902) called this form of mitosis *haplomitose*, but it seems questionable whether it is simpler than ordinary mitosis. Indeed, it is doubtful whether one can make a general statement that mitosis is usually simpler in Protozoa than in other organisms, though it is certainly much more diverse. (See Grassé's admirable review of the strange process of 'pleuromitosis' in certain Protozoa (1952, pp. 104-16).) The basic facts remain that chromosomes are concerned in the formation of new nuclei, and that these chromosomes multiply by longitudinal division.

THE INDIRECT ORIGIN OF CELLS FROM CELLS

History of the discovery

In the formulation of the cell-theory used in the present series of papers, the third proposition is this: 'Cells always arise, directly or indirectly, from pre-existing cells, usually by binary fission' (1948, p. 105). It remains to consider the indirect origin of cells from cells; that is to say, the development of a syncytium from a cell, and then of cells from the syncytium. The existence of syncytia, but not their development or transformation, has already been considered in Part III of this series (1952, pp. 177-83).

It was unfortunate that Schleiden (1838) chose a syncytium, the young endosperm, as his main subject of study when he was trying to find how cells develop. If he had chosen a tissue in which cells multiply by binary fission, it is scarcely possible that his ideas on the origin of cells would have been so erroneous. Through his influence the endosperm became a classical site for the study of the origin of cells.

Nägeli devoted much attention to cell-formation in endosperm and other syncytia. One would not suppose, from a study of his writings (1844, 1846), that binary fission was a more usual method of cellular multiplication. He performed a very useful service in demonstrating the error of Schleiden's views. The importance of his discoveries about the origin of cells in syncytia tends to be blurred for modern readers by the disproportionate emphasis he placed on his distinction between *freie* and *wandständige Zellenbildung*. In fact, however, he gave the first adequate account of the origin of cells in syncytia. He recognized that in endosperm and certain other sites there were numerous nuclei, not separated by cell-walls. (For his views on the origin of these nuclei, see above (pp. 451, 453, and 457).) The *Schleim* (protoplasm) lying between the nuclei now underwent a process of *Individualisierung* round the nuclei, and a *Membran* (cell-wall) was formed at the surface of each individualized portion of the *Schleim*. Thus, as many cells were formed as there were nuclei. Sometimes the newly-formed cells were spherical or nearly so, and free from one another and from the wall of the maternal syncytium; inevitably part of the syncytial protoplasm failed to be incorporated in the cells. This was *freie Zellenbildung*. In other cases (*wandständige Zellenbildung*) no protoplasm was

left out of the new cells, for the cell-walls were formed in immediate apposition to one another (or, in the external part of the syncytium, to the maternal cell-wall).

Rathke and Kölliker were the first to describe the origin of cells from syncytia in animals. I have already published a translation of Rathke's words (1952, p. 180). He remarks very tersely that in the embryo of Crustacea, nuclei are formed for the future embryonic cells before the cells themselves originate. He does not mention the mode of formation of the nuclei.

In his study of the development of cephalopods, Kölliker (1844) realized that the *Furchungssegmente* (blastocoines of Vialleton, 1888) were not separated from the yolk by any distinct boundary. He must have realized, therefore, that the *Embryonalzell* (nucleus) of one blastocone was not separated from that of the next by any membrane; or, to put it in other words, he must have understood that he was dealing with a syncytium. He knew that the nuclei duplicated themselves in the blastocoines, and that uninucleate cells (the *Furchungskugeln* or blastomeres) were budded off from their tips. It follows that he described the origin of cells from a syncytium, though this fact is obscured by his strange nomenclature, which I have already explained (1953, p. 418).

Incomplete cleavage is usually called meroblastic, but Remak himself (1852), when he introduced the term *méroblastique*, did not attach exactly this significance to it. For him, an egg was meroblastic if the embryo was formed from only a part of it: if the whole egg clove to convert itself into the embryo, it was holoblastic. It seems uncertain whether the egg of cephalopods would be meroblastic by Remak's definition, despite the fact that the cleavage-furrows do not pass right through it. The meaning of the term that is usual today appears to derive from Nicholson (1870, p. 217), who wrote of the development of the lobster, 'The ovum is "meroblastic", a portion only of the vitellus undergoing segmentation.'

The first really adequate description of the origin of cells from a syncytium in animals was given by Leuckart (1858, pp. 210-11), in his account of the development of *Melophagus* (Diptera Pupipara). He tentatively derived the nuclei of the early embryo from the germinal vesicle of the egg. He remarks that the development of the egg of insects conforms to the usual process of embryonic cell-formation, but 'A difference appears to me to exist here, only in so far as in insects the envelopment of the cell-nuclei with yolk-substance first occurs late, after the number of nuclei has already been considerably increased, while in other cases such an envelopment happens from the beginning, so that the division of the nuclei has then for a consequence, naturally and also constantly, a division of the yolk'. Robin (1862) noticed this method of cell-formation in various culicines; he called it *gemmation* and distinguished it from cleavage. He did not remark, however, on the presence of one nucleus in each of the cells formed by this process. Weismann confirmed Leuckart's findings by his studies of the development of *Chironomus* (1863a, pp. 112-13) and *Musca* (1863b, p. 163). He noticed the rising of the nuclei to the *Keimhaut-blastem* (blastoderm) in *Musca*, and compared it to the rising of air-bubbles

to the surface of water. When the blastoderm had separated itself off round each of these nuclei, the newly-formed cells multiplied by ordinary binary fission.

Comment

Syncytia that eventually resolve themselves into cells do not constitute an exception to the cell-theory as formulated in this series of papers. Particular parts of the body are often permanently syncytial. Not many groups of organisms are wholly syncytial, even in their somatic tissues. The belief that rotifers provide an example will not withstand critical examination, though many of their organs are wholly or partly syncytial (Martini, 1912; Nachtwey, 1925). The same applies to nematodes.

In the great groups of syncytial plants, the Siphonales, Cladophorales, and Phycomycetes, there is nearly always a periodical reversion to the haplocyte or diplocyte; that is to say, to the *cell* as defined in this series of papers (Part III, 1952). It will be recalled that the gametes of the Siphonales are generally flagellate cells; in *Vaucheria*, in which they are not flagellate, they are nevertheless cells. In the Cladophorales, asexual reproduction is in nearly every case by zoospores in the form of flagellate cells.

The two groups of Phycomycetes differ markedly in their reproductive processes. In the Oomycetes, asexual reproduction is generally by flagellate cells, sexual reproduction by the fusion of uninucleate protoplasmic masses from the antheridium with uninucleate ova, or, in the more primitive forms (Uniflagellatae), generally by the fusion of uninucleate flagellate gametes. In certain Zygomycetes, however, the cellular phase seems genuinely to have been lost. In *Pilobus crystallinus* the sporangiospores are in fact uninucleate, but in some other species each spore is multinucleate, so that asexual reproduction occurs without the intervention of a cellular phase. This applies, for instance, to *Rhizopus nigricans* and *Sporidinia grandis*. Now it is characteristic of sexual reproduction in Zygomycetes that the whole of the syncytial protoplasm of one gametangium fuses with that of another, with subsequent fusion of the nuclei in pairs. The new individual produced by this fusion proceeds to asexual reproduction (without any intercalated cellular phase) by forming multinucleate sporangiospores. The cycle is thus completed without the existence of a cell at any stage of the life-history.

Such forms as *Rhizopus* and *Sporidinia* are of exceptional interest to the student of the cell-theory. Their existence is a disproof that the theory is of universal application. It is to be remembered, however, that we can quote few similar examples in plants, and nothing at all that is even remotely similar in animals, except in certain Ciliophora. A discussion of the latter is reserved for a future paper in this series, which will be devoted to a consideration of the status of the Protozoa from the point of view of the cell-theory. (I have already treated the subject shortly (1948a).)

How has it come about in the course of evolution that the great majority of organisms consist largely of cells or at least are derived from and return to

cells in the course of their life-histories? This is one of the fundamental questions of biology, yet there is not very much that can be said in answer to it.

It is necessary to consider the somatic tissues separately from the gametes, because quite different causes appear to have been at work. In the case of the somatic tissues it is to be noted that a high degree of complexity of structure is never reached in a wholly syncytial organism. The repeatedly-quoted case of *Caulerpa* (Siphonales) is misleading, for the parts that superficially resemble the leaf, stem, and root of higher forms do not attain to anything approaching the degree of differentiation that their external aspect would suggest. It seems that organisms can more easily achieve differentiation in a cellular tissue than in a syncytial mass of protoplasm. This may perhaps be correlated with the obvious fact that synthesized substances are more easily localized in cellular tissues. It might perhaps be possible to devise experiments to discover something about the advantages an organism obtains by keeping its protoplasm in amounts small enough for each to be related to a single nucleus.

The reason why even a somatically syncytial organism nearly always has unicellular gametes is of quite a different nature. Why should not a higher animal, for instance, reproduce by syncytial gametes, like those of *Rhizopus*? Let us suppose that the nuclei of the syncytial gametes of such an animal were the immediate products of meiosis. They would necessarily differ among themselves in their gene-complexes. When karyogamy had occurred, a wide assortment of gene-complexes would be present in the embryo. Let us suppose, for example, that one of these complexes was such as to be potentially favourable to the survival of the organism, if present in nerve-cells. It will at once be evident that the nuclei derived from the zygote nucleus carrying that particular complex might be absent from the nervous system and present only in other tissues, in which it could not exhibit its beneficial effects. There would only be two ways of overcoming this barrier to the action of natural selection and therefore to evolution. One possibility would be to form a large number of uninucleate spores, each capable of developing into a whole organism carrying the same gene-complex in every nucleus of the somatic tissues. (This is exactly what some of the Zygomycetes do—and without wasting much time on vegetative growth at this stage.) A much simpler and quicker way would be to reproduce sexually by uninucleate gametes.

Natural selection can only act effectively on an organism that has the same gene-complex in the nuclei of all its somatic tissues; and that can only be achieved by periodical reversion to the unicellular state.

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Egg-capsule Proteins of Selachians and Trout

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SUMMARY

1. The material of the egg-capsules of selachians and the chorion of trout eggs has been examined by physical, chemical, and histochemical methods.
2. The material of the egg-capsule of selachians has been found to be a quinone-tanned protein.
3. The chorion of trout eggs is not quinone-tanned but its formation and chemical behaviour allies it with the invertebrate cuticular proteins rather than with the vertebrate keratins.

INTRODUCTION

WHILE the physics and chemistry of vertebrate epidermal proteins, the α - and feather-type keratins (Rudall, 1946), have been extensively investigated, the organic membranes surrounding the eggs of oviparous vertebrates have received very little attention. These latter structures, in contrast to the epidermal keratins, are extracellular secretions, analogous to the cuticles and egg-membranes of invertebrates. Two such membranes, very different in origin, the selachian egg-case and the chorion of trout eggs, have been examined and the results are reported here.

THE SELACHIAN EGG-CAPSULE

Between 1937 and 1938 Fauré-Frémiet and his co-workers investigated the structure and chemistry of the egg-capsules, and the anatomy and histology of the glands secreting them, in various selachians; and in the course of publication of their results they have given a full review of earlier work on this subject.

The egg-capsules are more or less rectangular in outline, with the corners prolonged into hollow tubes or horns. In detail the structure of the capsule wall differs in different species. The capsules from different species also differ in size. That of *Raja clavata*, the species occurring most commonly at Plymouth, is on average 7.5 cm. long (excluding the horns) and 5.7 cm. wide (Clark, 1922). Fauré-Frémiet (1938) described the structure of the capsule in *Scyliorhinus*, *Raja batis*, and *R. undulata*. In these, as indeed in many species, the wall of the capsule consists of three or four distinct layers. Each layer consists of a number of birefringent laminae, and each lamina is composed of parallel fibrils lying in the plane of the lamina, while in successive layers the fibrils are oriented approximately at right angles to each other, either parallel to or at right angles to the length of the capsule. In some species one of the inner layers may be alveolar. In *Raja* spp. the outer layer is much more

coarsely fibrous than the inner layers and in this layer the fibrils run longitudinally.

The anatomy and histology of the shell-gland which secretes the egg-capsule have been described by Filhol and Garrault (1938) in *Raja batis*, *R. miraletus*, *Mustelus vulgaris*, *Acanthias vulgaris*, and *Scyliorhinus canicula*. While there are differences between the species, they conform to the same general plan (figs. 1 and 2). The glands are swellings at the top of the oviducts. Within the gland it is possible to distinguish two regions; an upper, producing the albumen that surrounds the egg inside the capsule, and a lower, producing

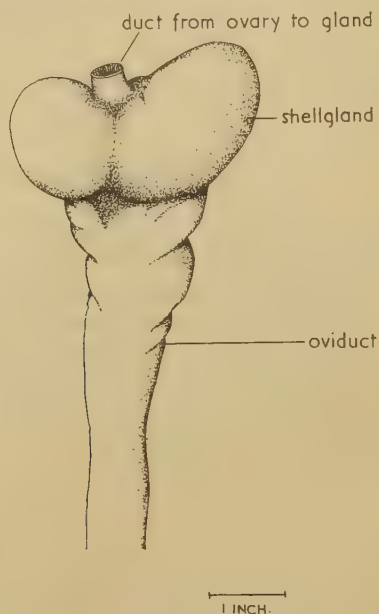


FIG. 1. Shell-gland from *Raja* spp.

granules that form the capsule. In *Raja batis* the portion of the gland concerned with the production of the capsule is further divided into two parts: (1) a part composed of long branching tubes lined by granule-producing cells along their whole length; these tubes open into troughs between a number of long parallel ridges on the internal wall of the shell-gland; and (2), the rest of the capsule-producing portion of the shell-gland; this is composed of groups of granule-cells, discharging by a duct into tubes lined by mucous cells. These tubes themselves discharge through pores in the lower portion of the gland.

Fauré-Frémiet and his co-workers assumed with Krukenberg (1885) that, as the material of the capsule is a protein containing sulphur and resistant to most chemical reagents, it is a keratin. Filhol and Garrault (1938) therefore called the granules concerned in the formation of the capsule 'prokeratin granules'. Fauré-Frémiet and Garrault (1938) have described a method for obtaining the granules in a condition suitable for chemical analysis and Fauré-

Frémiet and Baudouy (1938) analysed, for nitrogen and sulphur, the 'prokeratin granules' and the different layers of the capsule. The average sulphur content of the 'prokeratin granules' was found to be 1.4% and of the internal, middle, and external layers, 1.19%, 1.08%, and 0.85% respectively compared with the sulphur content of human hair of 5% (Block, 1938).

Filhol and Garrault (1938) comment on the similarity of the histology of the shell-gland of selachians and the glands in the foot of *Mytilus* concerned in the production of the byssus. Brown (1952) showed that the byssus of *Mytilus* is quinone-tanned, and in view of this similarity in the histology of the glands and in the appearance of their products, it seemed desirable to examine the possibility of quinone-tanning in the egg-capsule of selachians.

Material

Most of the material used was collected through the co-operation of the skipper of a Newlyn fishing boat, who preserved in 80% alcohol the eggs and

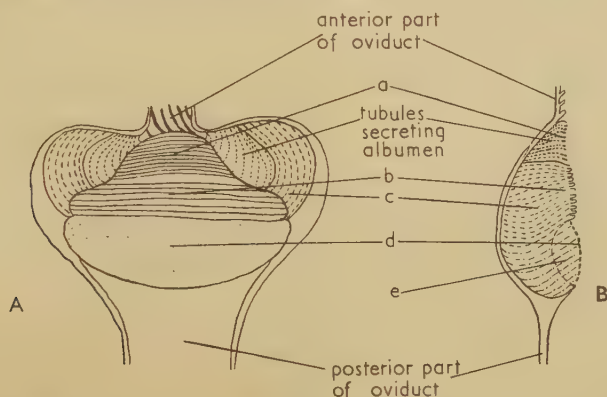


FIG. 2. Diagram of shell-gland from *Raja* spp. A, lateral longitudinal section. B, dorsoventral longitudinal section. *a*, lips through which albumen coat of egg is secreted; *b*, transverse lips concerned with formation of capsule; *c*, tubules carrying products of cells producing 'prokeratin'; *d*, pores concerned with formation of capsule; *e*, area in which tubules are lined by mucous cells.

shell-glands from ripe females found when gutting the fish. In such material it was not possible to identify the species with any certainty. The material thus collected was examined by methods which permit the determination of the types of linkage between protein molecules in any particular structure (Brown, 1950). On these depend its mechanical and chemical properties.

Results

Material from fully-formed capsules and material from half-formed capsules removed from the oviducts was tested, and the capsule wall in both cases was separated into a dark outer portion and a white inner portion before testing. The properties of the two portions were examined separately. Before the material was examined it was washed repeatedly in distilled water to remove the preserving alcohol.

When boiled in distilled water all specimens contracted in length and width

and increased in thickness. This result is to be expected from material composed of fibrillar laminae with a periodic change in the fibril direction through 90° if boiling water hydrolyses some of the electrovalent bonds between the protein molecules forming the fibrils, allowing them to contract. In dilute hydrochloric acid in the cold, both layers of mature and partly formed capsules decrease in length and width and increase in thickness, curl up and become rubbery. Dilute sodium hydroxide solution in the cold has no obvious effect on the capsule material, but on boiling the material completely dissolves. All these results confirm the presence of weak electrovalent bonds holding the material together.

Alkaline sodium sulphide solution, which dissolves vertebrate keratins by breaking the disulphide bonds, has little or no effect on either layer of the fully formed capsule, but it causes the outer layers of the partly formed capsule to swell and soften, and completely dissolves the inner layers. These results suggest that disulphide bonds are important in holding the protein molecules together in the early stages of formation of the capsule, but in the fully formed capsule some further linkage of the material takes place supplementing the disulphide bonds and preventing it from dissolving in alkaline sodium sulphide solution. Conceivably, as in *Mytilus*, this further linkage is a quinone-tanning process.

Ferric chloride tests for the presence of orthodiphenols were negative on fully formed capsules taken from the sea, but on partly formed capsules they were strongly positive both for inner and outer layers.

Sections of the shell-gland were cut and stained for the presence of quinones by the argentaffin (Lison, 1936) and diazo techniques (Danielli, 1947). Both these gave positive staining of the 'prokeratin' granules. While the granules in the cells of the gland did not stain very darkly, the granules secreted into the tubes of the gland gave a very intense reaction. The positive ferric chloride reaction of the immature capsule and positive diazo and argentaffin staining of the 'prokeratin' granules, both indicate the presence of a polyphenol which could participate in tanning the protein of the capsule.

Portions of inner and outer layers of a fresh, partly formed capsule were incubated with *l*-tyrosine solution at pH 8.0 for 12 hours at 37° C. to test for the presence of polyphenol oxidase. Controls were set up with potassium cyanide added to the solution. The dark outer portion of the capsule became very dark brown, and the solution brown; the inner white portion slightly brown with some slight browning of the solution. In both controls there was no change in colour. These results indicate the presence of a polyphenol oxidase which might serve to oxidize the polyphenol present in the capsule to a quinone capable of tanning the material.

Conclusion

From these results it would seem to be incorrect to describe the protein of the egg-capsule of selachians as a keratin, since it differs considerably in its behaviour from the true keratins, and it is better regarded as a sclerotin in

Pryor's sense (1940). It is interesting that this vertebrate protein the secretion of which has much in common with the secretion of invertebrate structural proteins should also have chemical affinities with invertebrate structural proteins. The presence of disulphide bonds indicates also a relation to the keratins but important disulphide bonds are also present in such invertebrate structures as the cuticle and hooks of cestodes (Crusz, 1948) and in the carapace of *Limulus* (Lafon, 1943).

Regarding the mechanism of orientation of the fibrils in successive laminae and the change of orientation through 90° , nothing can be said with certainty. The transverse lips (*b* in fig. 2, B) of the shell-gland would seem fitted to lay down fibres transverse to the long axis of the egg-capsule and the pores (*d* in fig. 2, B) of the gland fibres oriented at 90° to these; but the cause of alternate orientation in successive layers is still obscure.

THE CHORION OF TROUT EGGS

The eggs of salmon and trout are enclosed in a tough membrane, the chorion, which is analogous to the capsule of selachian eggs, but in the salmon and trout is secreted by cells in the ovary and not by a gland of the oviduct.

Young and Inman (1938) studied the chemistry of the chorion of *Salmo salar*. They found that it was extremely resistant to normal solvents in the cold, but dissolved in 1% sodium hydroxide solution and hydrochloric acid at 100°C . Trypsin had no effect, but pepsin digested the chorion. The amino-acid content was also determined by these workers, as follows:

	<i>Salmo salar</i>		Human hair (Block, 1938)
	%	%	%
Total N . . .	15.20	15.32	14.9
Arginine . . .	5.72	5.85	8.0
Histidine . . .	1.23	1.28	0.6
Lysine . . .	3.54	3.47	2.5
Tyrosine . . .	5.12	5.12	2.9
Tryptophane . . .	1.42	1.42	0.7
Cystine . . .	1.79	1.89	14.7
Glucosamine . . .	1.04		

A few swelling experiments were made on the chorion of trout eggs (*S. irideus*). Boiling distilled water caused an anisometric contraction and thickening of the chorion, indicating the presence of weak electrovalent linkages, hydrolysed by boiling water, and predominant orientation of protein chains in the plane of the chorion with some preferred orientation with respect to axes in the membrane. Neither sodium hydroxide solution alone nor with sodium sulphide after prolonged treatment in the cold dissolves the chorion, but in both cases strips of the chorion contracted in one direction and elongated in the other, becoming very fragile and easily broken on handling. These dimensional changes again suggest the presence in the chorion of fibrillar

proteins oriented in the plane of the chorion with some preferred orientation. Calcium cyanate, lithium cyanate, and hydrochloric acid all cause the chorion to contract slightly and become rubbery. Sodium hypochlorite solution was the only reagent, besides boiling acids and alkalis, that dissolved the chorion.

Sections of the chorion, stained for —SH and —S—S— groups by the technique of Chèvremont and Frédéric (1943), indicate the presence of —S—S— groups in the chorion. Diazo and argentaffin staining for polyphenols gave negative results.

Conclusion

The material of the chorion differs in chemical behaviour from that of α - and feather-keratins. Young and Inman showed that the amino-acid content also differs considerably from that of true keratins; and it is not a quinone-tanned protein, as is the material of the selachian egg-capsule. Block (1938) classifies it as a pseudokeratin which suggests that it has at least some keratin characteristics, but its mode of secretion and its chemical behaviour are more allied to invertebrate cuticular materials, though its exact nature has not yet been determined.

Part of this work was done at the Marine Biological Laboratory, Plymouth, to the Director and Staff of which I am most grateful for the facilities given me. I am particularly grateful to Mr. G. A. Steven for advice on the obtaining, handling, and identification of material. I am also indebted to Professor J. F. Danielli for much advice about histochemical techniques and to Dr. L. E. R. Picken who gave invaluable suggestions, criticism, and advice through all stages of the work.

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A Study of the Round Pigment Cells in the Uterus of the Ewe

By R. HADEK

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With one plate (fig. 1)

SUMMARY

The round pigment cells in the uterus of the ewe have been found to contain, in addition to melanin granules, *Abnützungspigmente* (pigments of 'wear and tear'). On account of their ability to absorb and store material from the neighbouring tissue, the cells are regarded as phagocytes.

INTRODUCTION

THE mucosa of the bicornuate sheep uterus like that of other ruminants consists of glandular and non-glandular areas (fig. 1, A). The non-glandular areas, or caruncles, are button-like prominences in the sheep, which are raised above the uterine surface and in some specimens appear jet black. The black colour is due to a large number of melanocytes in the endometrium. They are predominantly connective tissue structures with a rich blood-supply. The number of caruncles has been described as alternating between 60 and 120 (Grant, 1933). Placentation in the sheep is always localized in these caruncular areas. Slightly branching tubular glands are located in the inter-caruncular areas, so that the two zones differ both functionally and structurally. The diameter of the caruncles is widest during early dioestrus and narrowest during late dioestrus and the beginning of prooestrus. During oestrus, metoestrus, and early dioestrus, oedema of the uterine stroma and increased vascularity with congestion of the uterine capillaries is noticeable (McKenzie and Terrill, 1937). In the same period, folding of the epithelial surface takes place and increased coiling and growth in the height of the glandular cells is visible (McKenzie and Terrill, 1937). The uterine epithelium of the non-pregnant sheep undergoes cyclical alteration during the oestrous cycle, being lowest in late dioestrus and early prooestrus, increasing in height in oestrus and reaching a peak in metoestrus and early dioestrus. A lymphocytic migration through the uterine epithelium takes place during the metoestrous and dioestrous part of the cycle, with the highest concentration during early dioestrus.

LITERATURE

Many accounts have been given of the structure of the uterus in the sheep (see review by McKenzie and Terrill, 1937).

The occasional presence of melanotic pigment cells in the uterine mucosa of German sheep breeds was first noted by Bonnett (1880), and later by Kazzander (1890). Assheton (1906) and Grant (1933), who described the continuous presence of black pigment cells in the uterine caruncles of the

Scottish Blackface ewe, divided them into two groups, melanoblasts and round cells. While Kazzander (1890) and Grant (1933) described the features of the melanoblasts, no attempt was made to study the round cells in detail.

MATERIAL AND METHODS

The genital tracts of 60 ewes were obtained for the purpose of this study. Of the 60, 12 were in the anoestrous, 12 in the prooestrous, 12 in the oestrous, 12 in the metoestrous, and 12 in the dioestrous phase of the cycle. The major part of the material (50) was obtained at the Glasgow Abattoir from freshly slaughtered sheep and the rest recovered through hysterectomy of experimental ewes. The correct phase of the cycle in the abattoir ewes was estimated from the gross anatomical and microscopical appearance of the genital tract and ovaries, while in the case of the experimental ewes the mating attempts of a teaser ram served as the criteria for heat, and other periods of the oestrous cycle were counted from that time.

Staining methods used for morphological observations included haematoxylin-eosin, Gordon Sweet's and Laidlow's methods for reticular fibres, Masson's trichrome, Weigert for elastic fibres, and van Gieson for collagen staining.

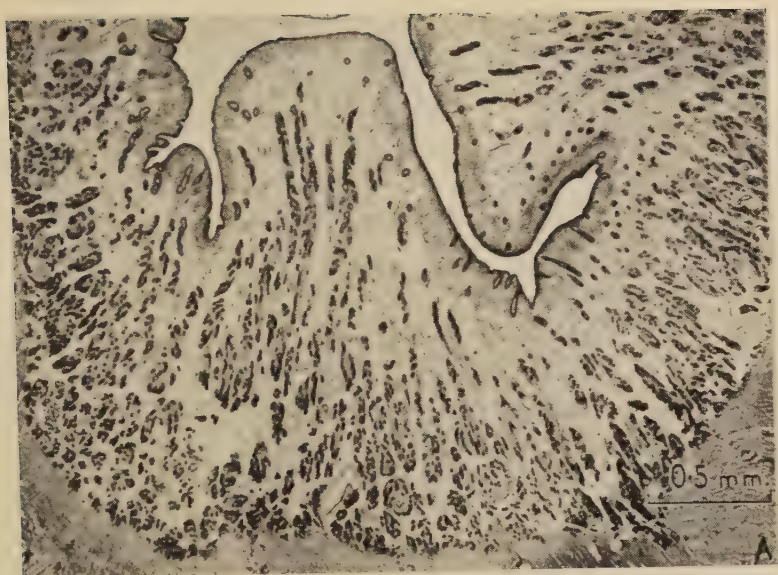
The histochemical techniques performed can be divided into the following groups:

Iron. Prussian blue test and Turnbull's blue for ferric, ferrous, and organic (masked) iron (Glick, 1949); Lavalley's hydroxyquinoline test for iron, Humphrey's dinitroresorcinal test (Glick, 1949), and the haematoxylin-lake method (haemosiderin iron—Pearse, 1953).

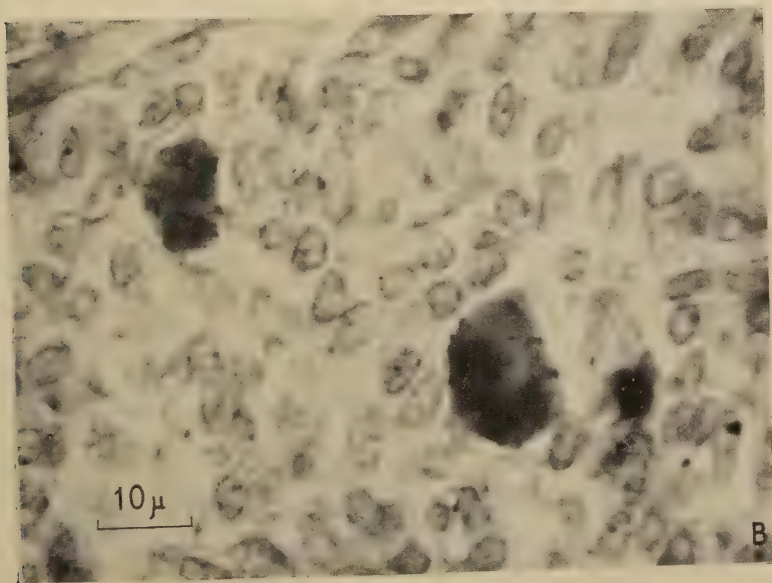
Polysaccharides. Periodic acid Schiff (henceforward abbreviated to PAS) according to McManus, (1946; 1948) and Hotchkiss (1948 *a* and *b*). Only occasional sections were counterstained with haematoxylin. If staining with PAS was positive, sections were treated with (1) diastase (B.D.H.) for 30 minutes at 37° C; (2) hyaluronidase (Benger 'Hyalase'), 1,000 units per 100 c.c. of buffered solution for 2 hours at 37° (Hale, 1946); and (3) with warm methanol-chloroform mixture for 12 hours (Leblond, 1950). If controls (1), (2), and (3) were negative (that is to say, if the material after the respective treatment still continued to react with PAS), further sections were stained with (4) Southgate's mucicarmine (Cowdry, 1948), (5) toluidine blue (Gomori, 1952) and (6) celestin blue (Gomori, 1952).

Nucleic acids. For the staining of ribonucleic acids (RNA) the method of Unna-Pappenheim (Brachet, 1940) and methylene blue staining at pH 3, 5, and 7 was used. Control specimens were treated with Fisher's (1953) mineral acid extraction method. For the staining of desoxyribonucleic acid (DNA) the method of Feulgen (Feulgen and Rossenbeck, 1924) was used and control specimens treated according to the method of Oster and Mulinos (1944).

Lipids. The modified Feulgen plasmal reaction (Cain, 1949), Sudan IV staining according to Herxheimer technique (Conn, 1946), and haematoxylin staining in the Smith-Dietrich method (Conn, 1946); Baker's (1946) acid



A, cross section of sheep endometrium, showing caruncular and glandular areas. Haematoxylin/eosin



B, round pigment cells in sheep endometrium. Haematoxylin/eosin

haematein test and pyridine extraction method; Schultz's method for cholesterol (Gomori, 1952).

Pigments. A number of the previously described reactions which were applicable; further, washing with saturated acids, alkalis and acidified permanganate; and the Dopa reaction according to Gomori (1952).

Enzymes. The methods for alkaline and acid glycerophosphatase according to Gomori (1952), with an incubation period of 1-2 hours. The treatment of controls was identical with those advocated by him.

The Round Pigment Cells of the Uterus

The round pigment cells described in this study were found throughout the endometrium and occurred in greatest numbers in the caruncles (fig. 1, B). They were present in fairly large numbers in each phase of the cycle with the exception of anoestrus, when they were infrequent or absent.

The cells varied from 8.5μ to 25.0μ in diameter. In the unstained, unfixed sections they were easily distinguished from the surrounding material on account of their general yellow colour. In unstained paraffin sections of material fixed by a colourless fixative (e.g. mercuric chloride) the cells showed an identical colour due to the presence of yellow globules in the cytoplasm. In addition to these globules, and often within them, a number of fine black pigment granules occurred in some of those cells which lay immediately beneath the uterine epithelium.

In haematoxylin-eosin sections the basophil nucleus as a rule was clearly visible in the smaller cells whose cytoplasm contained only a few granules, whereas it was usually obscured in the larger cells by the spherical globules. These small cells looked similar to connective tissue cells and it was only the presence of the yellow granules which made it possible to recognize them as pigment cells. The cytoplasmic globules, which stained yellow or reddish-brown with eosin instead of the red colour of the neighbouring cells, almost completely occupied the cytoplasm.

Masson's trichrome stain stained the cytoplasmic globules brown-black; with silver stains the presence of a number of minute granules was noted in them.

The histochemical reactions of the cytoplasmic globules were as follows:

Inorganic iron. Negative.

Organic (masked) iron. Negative.

Polysaccharides. The periodic acid Schiff reaction was invariably positive. In well-stained specimens one could easily differentiate between the dark red globules and much fainter cytoplasmic reaction. The intensity of the staining reaction was increased after diastase and hyaluronidase treatment but it remained unchanged after overnight incubation in methanol-chloroform. Attempts to obtain metachromasia with toluidine and celestine blue failed.

Nucleic acids. The nuclei of the cells gave positive staining with Feulgen's reaction and with methyl green (Unna-Pappenheim). The cytoplasm of the

round cells showed a general red colour with Unna-Pappenheim staining and in it the cytoplasmic globules appeared dark red.

Lipids. In frozen sections the yellow globules stained strongly with Scharlach R, in the Herxheimer method, and with haematoxylin in the Smith-Dietrich and Baker's acid haematein tests. After pyridine extraction the globules did not stain with Baker's acid haematein. Feulgen plasmal was negative and so was Schultz's cholesterol test. The globules stained with Nile blue and the dye was not extracted by hydrogen peroxide. In paraffin-embedded specimens, the globules which reacted strongly with PAS stained well with Sudan black B, with Scharlach R (Herxheimer method), and the strength of the staining reaction only diminished after acetone treatment of the sections. If the sections were treated with fat solvents after staining they became decolorized, but the globules could be restrained with the same dyes.

Pigments. Two types of pigments were present. One, which was orange-yellow, occurred in the cytoplasmic globules and stained with PAS, &c., and with the above lipid colorants. The other consisted of the minute black particles which were occasionally encountered in the cytoplasm of the cells and sometimes in the orange globules. The dopa reaction was consistently negative.

Treatment of sections in dilute acids and alkalis had no effect on the minute black pigment granules in the round cells. Acidified permanganate or peroxide did not have any visible effect on the granules or on the cytoplasmic globules.

Enzymes. No phosphatases were present.

DISCUSSION

The round pigment cells were found to be of different sizes and to contain varying amounts of pigment. The smaller ones in fact contained very little and were found to resemble connective tissue cells. From this it is supposed that the cells are of connective tissue origin and that their cytoplasmic content is gradually acquired during their expansion.

Although the larger part of the cytoplasm was occupied by a pigment, there were minute melanin granules among them also. The dopa reaction, however, proved negative and it was therefore accepted that the melanin was only acquired and not produced by the cells. This acquisition of melanin was easy, since the surrounding tissue was very rich in melanocytes which were continuously breaking down.

With regard to the yellow cytoplasmic pigment which occupied practically the whole cytoplasm of the cells, it was found that this was PAS positive and contained a lipid which was completely insoluble in lipid solvents. The lipid-containing pigments, according to Lubarsch (1902) and Ciaccio (1915), can be divided into two groups, namely, (i) the substances which lose their lipid reaction after treatment with sulphuric acid or ammonia, and (ii) *Abnützungspigmente* (pigments of 'wear and tear') which do not. Since the pigment did not lose its reaction after these treatments, it was an *Abnützungspigment*, which was absorbed from the neighbouring tissue like the melanin granules. The round pigment cells may therefore be regarded as uterine phagocytes.

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The Distribution of Hatching within the Cyst of the Potato Root Eelworm, *Heterodera rostochiensis*

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SUMMARY

1. A serial-sectioning technique is described for cysts of the potato root eelworm. The distribution of hatching among eggs contained in the cysts is investigated by this technique.
2. It is shown that there are significant differences in the numbers of larvae emerging from different parts of the cyst, i.e. hatching is not random. There is a tendency for eggs near the cyst wall to hatch sooner than eggs nearer the centre of the egg mass.
3. The position of eggs in relation to the natural openings of the cyst, at the neck and vulva, is apparently without influence on the distribution of hatching.
4. Although the cyst wall is permeable to the hatching stimulant it is argued that the hatching pattern is more likely to be due to a gradient of oxygen tension within the cyst.

INTRODUCTION

IT is well known that larvae are stimulated to emerge from cysts of the potato root eelworm by the action of a substance diffusing from the roots of the growing host-plant (Triffitt, 1930b). Under laboratory conditions once hatching has begun the number of larvae emerging each day reaches a maximum and then decreases to zero even in the presence of fresh root diffusate. If the same cysts are re-stimulated after an interval hatching recommences, showing that the original cessation was not due to the cysts being empty but to some other cause.

Ellenby (1946b) showed that cysts with a small puncture in the wall produced roughly twice as many larvae as unpunctured controls before hatching ceased. Pointing out that the mere presence of another exit cannot itself cause larvae to hatch if they are not ready to do so, Ellenby concludes that the cessation of hatching must be brought about by inhibition. He postulates the production, perhaps by the larvae themselves, of an inhibitor which, on reaching a critical concentration, inhibits further hatching. The puncture provides another exit for the inhibitor which therefore takes longer to build up to the critical concentration. Ellenby suggests that if this is the correct explanation the eggs constitute an ecological community, the behaviour of individuals in the community affecting the behaviour of the others.

If the eelworm cyst is, in fact, an ecological unit, hatching from it may be governed by definite rules. The present paper reports the results of an investigation to determine whether hatching is random or whether the position of eggs within the cyst affects the order in which they hatch.

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MATERIALS AND METHODS

The cysts used in this work were collected from the roots of the potato, variety 'Redskin', after most of the soil had been removed. The cysts were considered to be full at the start of the work (i.e. no larvae having emerged), but the method of collection does not exclude the possibility of the inclusion of an occasional old cyst from which some emergence might have taken place. All cysts were more than two years old when the work began and the larvae are assumed to be capable of hatching when stimulated. The cysts were stimulated by a modified single-cyst technique (Ellenby, 1943) with potato root diffusate prepared either directly from the roots of a growing plant or from a sample of concentrated hatching factor (Calam, Raistrick, and Todd, 1949). Emerging larvae were counted and removed daily, fresh root diffusate from the same stock being added if necessary. In the majority of cases hatching was stopped when between 40 and 150 larvae had emerged.

The distribution of empty egg cases within the cyst was investigated by cutting serial sections by the technique described below.

Sectioning technique

Owing to the hardness and brittleness of the cyst wall, ordinary wax embedding techniques were a failure. Accordingly, the cyst wall was partially dissolved in sodium hypochlorite (Smedley, 1936); a 1:9 dilution of commercial 'Milton' was found most convenient. There is a good deal of variation in the thickness of the cyst wall which does not appear to be correlated with cyst size. Owing to this variation in thickness no definite time for the Milton treatment can be stated, but 60 minutes is a reasonable average. The cysts are examined under a binocular microscope and transferred to the fixative when the eggs are easily visible through the wall. Unstimulated cysts are soaked in distilled water for 2 or 3 days before treatment. Stimulated cysts are treated immediately after hatching ceases, but if this is inconvenient they are kept in distilled water until sectioning treatment begins.

The cysts are fixed in Duboscq-Brasil's fluid, which is highly penetrating and very suitable for animals with a cuticle; it is warmed to about 40° C to increase penetration. When all cysts have been in the fixative for 20 minutes or more (periods up to 60 minutes are not harmful) they are transferred to 70% alcohol for dehydration. Milton treatment and fixation can be carried out in the 'single cyst' Petri-dishes but dehydration and subsequent stages are best done in Stone's trays (Goodey, 1949) or in coverslip dishes (Fenwick, 1943).

After 30 minutes the 70% alcohol is replaced by 90% and after a further 30 minutes this is replaced by 95% for 15 minutes. To avoid the hardening effects of absolute alcohol the cysts are cleared in methyl benzoate to which celloidin has been added. This enters the cysts and keeps the contents in place during sectioning. To ensure complete impregnation the cysts are usually left in methyl benzoate / celloidin overnight. (If Stone's trays are used, the plastic of which they are made should be tested to see if it reacts with methyl benzoate.)

The cysts are next transferred from methyl benzoate / celloidin to benzene (15 minutes), a benzene / paraffin wax mixture (30 minutes), and finally to paraffin wax of melting-point 54°C , in which they are left overnight.

Porcelain paint cells ($18 \times 18 \times 10\text{ mm}$) were found to be better than watch-glasses for blocking cysts. Each cell is filled with molten wax and a cyst is transferred to it by using a cold mounted needle. A warmed needle brought close to the cyst causes it to fall into the cell where it is orientated under a low power binocular and its position marked while the wax is semi-solid. Sectioning is carried out in the normal way with the Cambridge rocker microtome.

Sections were cut at $20\text{ }\mu$; as the approximate size of eggs in the cysts used was $95 \times 40\text{ }\mu$, no full egg or empty egg-shell appeared whole in the sections. The distribution of empty eggs, i.e. of hatching, could therefore only be studied by noting the position within the cyst of the cut pieces of empty egg-shells consequent upon sectioning. These are referred to in the text as *fragments*. As hatching from an egg does not cause the shell to break up and pieces of full eggs contain pieces of larva, the *fragments* of empty egg-shells are easily distinguishable.

Distribution of fragments

Essentially, the cyst was divided into arbitrary regions and the hatches compared by noting the numbers of fragments in each.

As shown in fig. 1, A, the cysts were divided into three regions, the two *ends* and the *central region*. The ends each consisted of five sections; each is therefore a saucer-shaped piece of cyst wall containing eggs none of which is farther than $100\text{ }\mu$ from some part of the wall. When the ends contain the neck or vulva owing to the orientation of the cyst, they are known as *neck end* and *vulva end* respectively. The central region contains a variable number of sections according to the size of the cyst.

Drawings of the outline of each cyst section were made on squared paper by using a squared eyepiece, and the egg fragments drawn in as accurately as possible. A circle of known radius was inscribed in the centre (as determined by eye) of each section of the central region, dividing it into an inner zone or *core* and an outer *peripheral zone* (fig. 1, A and B). The series of drawings made for one cyst is shown in fig. 2.

Cysts were sectioned in one of two ways: either the plane of section was at right angles to the axis of the cysts, thus including the neck and in most cases the vulva in the ends, or the plane of section was parallel to the axis, the two apertures then being incorporated in the central region.

Although the neck will always be part of the end or central region according to orientation, the position of the vulva is not so easily defined, as the vulva is offset from the longitudinal axis of the cyst. An 'end' as defined is a shallow bowl of maximum depth $100\text{ }\mu$. Measurements of 70 cysts from the same stock as those used in the work showed that in 24 cases, had the cysts been sectioned transversely, the vulva would have been excluded from the bowl.

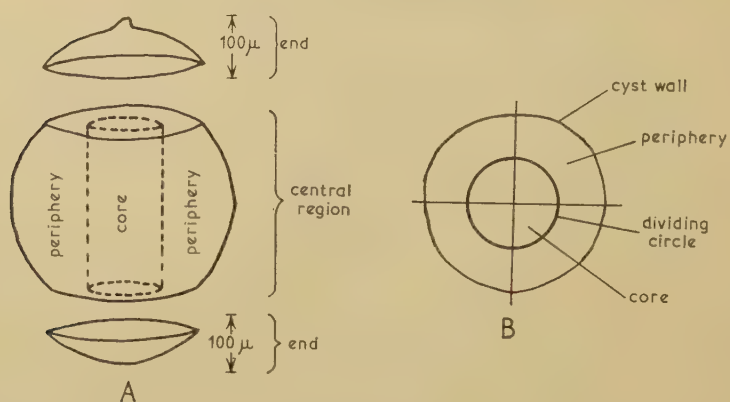


FIG. 1. A, subdivision of cyst into regions. B, division of each central region section into core and periphery.

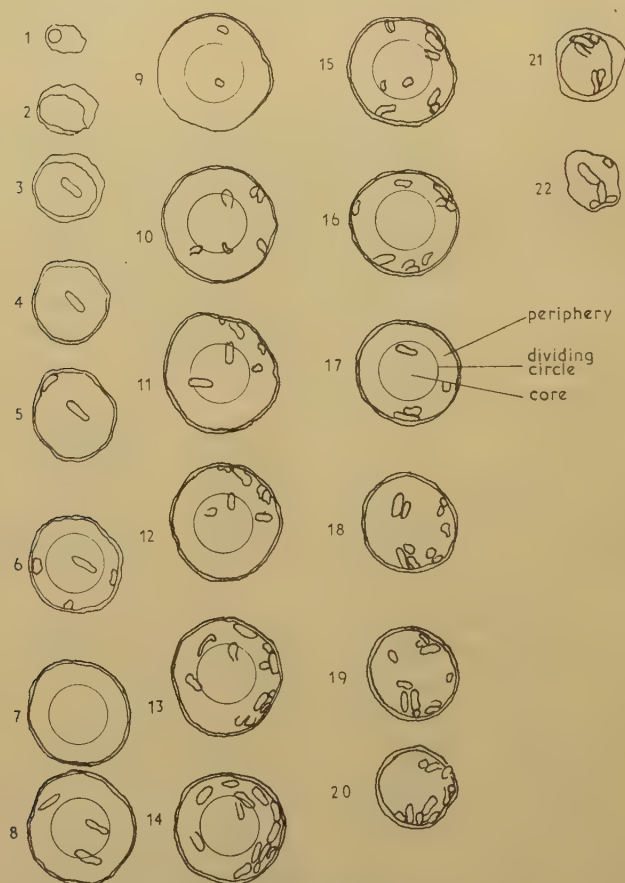


FIG. 2. Distribution of fragments in a series of sections from a transversely sectioned cyst.

Estimation of hatches from cyst regions

The areas of all sections were measured with a planimeter. As all sections are of uniform thickness ($20\ \mu$) and almost cylindrical, their volumes are proportional to their areas. Dividing the total number of fragments present in a particular region by its total area gave the hatch for the region as so many fragments per unit volume (f/vol).

Since eggs are not spherical, any comparison based on the numbers of egg fragments will be invalid if the eggs are not randomly arranged. Further, it is also necessary to know how accurately the number of fragments represents the number of larvae hatched. Both these points were investigated.

Fragment-larvae relationship and random arrangement of eggs

In fig. 3, A the relationship between fragments and larval emergence is plotted against cyst size for 51 cysts sectioned transversely. In fig. 3, B the same relationship is presented for 21 cysts sectioned longitudinally. (The point 154, 8.09 is omitted from the analysis since with an egg size of approximately $100\ \mu$ and section thickness $20\ \mu$ the ratio 8.09 is impossible. The most probable explanation is that this was an old cyst from which some hatching had occurred before it was collected.) Regression analysis shows that there is no significant tendency for the fragment/larva ratio to vary with change in cyst size in either case ($p < 0.1$ in both cases). The regression coefficients and their standard errors are presented in table 1.

There is, however, considerable variation in the fragment counts. For example, for cysts with 80 larvae hatched the counts vary between 150 and 300. Some of this variation might be due to errors in counting but this cannot be wholly responsible. It was considered that variation in cyst size might affect the fragment counts for if there is a large hatch from a small cyst the fragments will be crowded together and difficult to distinguish. The possibility was investigated.

The volumes of 72 cysts, assuming them to be spherical, were calculated and larval hatch per mm^3 and fragments per mm^3 were evaluated. The former were arranged in ascending order of size in groups of six and the variance of fragment densities for each group was evaluated. In fig. 4 variance is plotted against mean larval hatch density for each group. There is an obvious tendency for the variance to increase as larval hatch density increases. Fragment counting is accurate for fairly low larval hatch densities but where larval hatch density is high there is considerable error. It was for this reason that hatching was stopped, in the majority of cases, when between 40 and 150 larvae had emerged (p. 496).

Many hundreds of sections were examined in the course of the work. At no time was there any evidence that the eggs were not randomly arranged. This is confirmed by the mean values of the fragment/larva ratio for the two groups of cysts described above; for cysts sectioned transversely the mean value is 2.26, and for longitudinally sectioned cysts 2.44. Cutting sections in two planes at right angles therefore has little apparent effect on the numbers

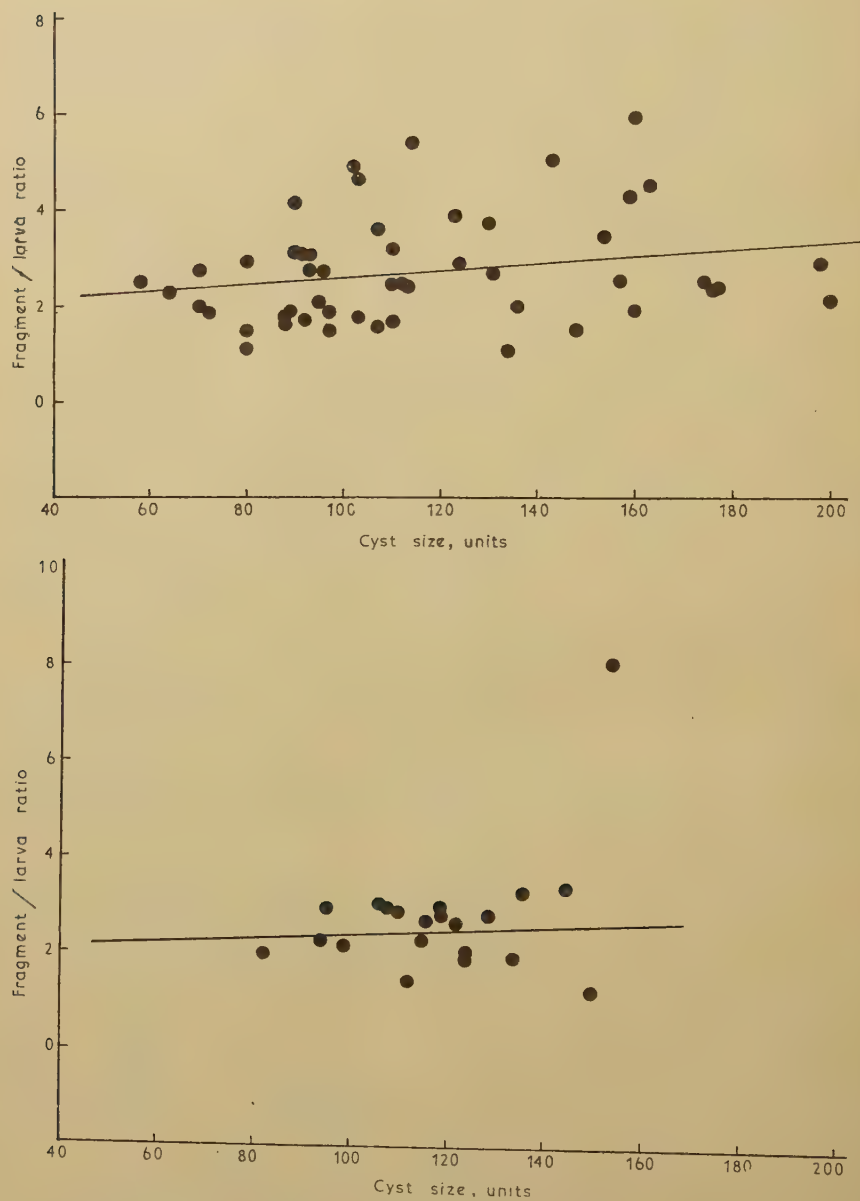


FIG. 3. A, fragment/larva ratio and cyst size for cysts sectioned transversely. B, fragment/larva ratio and cyst size for cysts sectioned longitudinally.

of fragments produced. Arrangements giving equal numbers of fragments in two planes at right angles are possible; there was, however, no evidence of any such arrangement and it is considered reasonable to assume that eggs are randomly orientated.

TABLE I

Fragment/larva relationship and cyst size. Regression coefficients and standard errors of regression

	Regression coefficient	Standard error of regression	<i>t</i>	<i>p</i>
Cysts sectioned transversely.	+0.007	±0.004	1.59	>0.1
Cysts sectioned longitudinally.	+0.005	±0.007	0.73	>0.4

If hatching from the cyst is random the fragment density will be the same in all regions. If, however, hatching is not random, fragment densities will be

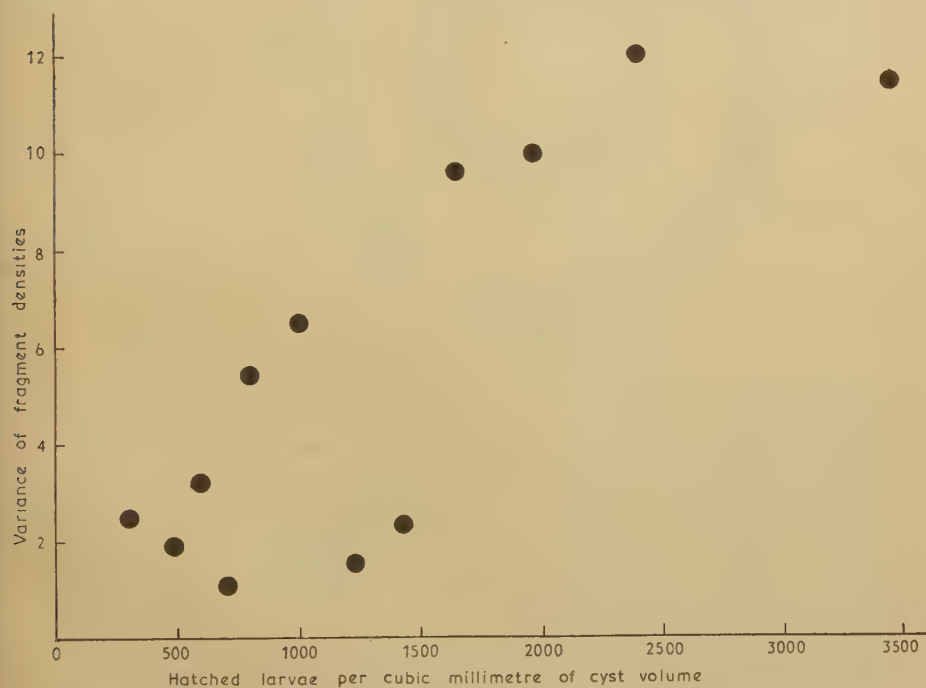


FIG. 4. Variance of fragment densities and larval hatch density.

highest where hatching is greatest and the fact that errors in counting will also be greatest there (fig. 4) will tend to minimize the differences between regions.

The mean values of the fragment/larva ratio are rather small. The thickness of the sections and the size of eggs suggest that the mean values should be in the region of 3.50. Presumably the difficulties of counting fragments when their densities are high accounts for the low values. Further, the ease with

which fragments are missed probably varies inversely with their size. Eggs cut in L.S. or near L.S. give two or perhaps three large fragments while eggs cut in T.S. give four or five much smaller fragments. While all of the three larger fragments may be counted, some of the latter may be missed, thus lowering the value of the ratio.

Sections were assumed to be cylindrical and their areas to represent their volumes. They are in fact most like cylinders near the centre of each group and least like them at the ends. Volume estimates based on area will be too large and hatch estimates therefore too small, the errors being greatest at the ends. As the core of each central region section is cylindrical the errors in

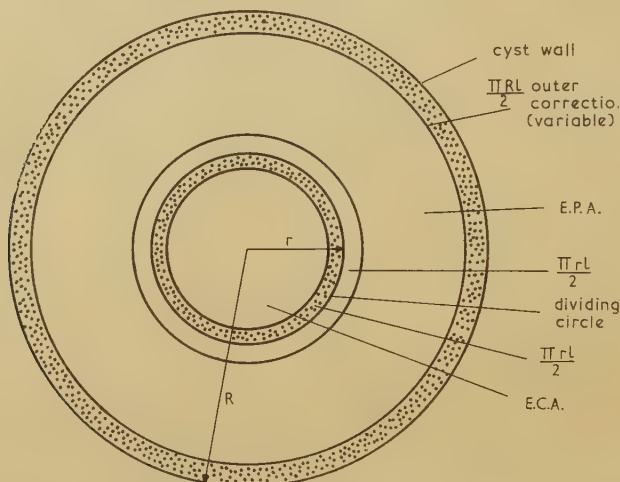


FIG. 5. Diagram of area corrections. E.P.A. and E.C.A. are the effective peripheral and core areas respectively.

the central region will affect the periphery only. If hatching is not random, the differences will clearly be affected by this assumption.

Some of the egg fragments lie on the line dividing the periphery of central sections from the core. As the core is smaller than the periphery in the vast majority of cases, the number of fragments it *could* contain is less than the maximum for the periphery. If the border-line fragments are ignored the core hatch estimate will be affected to a relatively greater extent. Some correction was therefore necessary.

The effect of ignoring the border-line fragments is compensated for by subtracting from the areas of both core and periphery an amount equal to $\frac{1}{2}\pi r l$, r being the core radius and l the mean fragment length. This represents an area in which the centres of eggs cannot lie without the eggs projecting over the line dividing periphery and core. An amount equal to $\frac{1}{2}\pi R l$, where R is the section radius, is also subtracted from the area of each periphery and from the area of each end section; this represents a region at the outer boundary of each section in which the centres of eggs cannot lie without the eggs projecting through the cyst wall.

Fig. 5 shows the area corrections for a central region section.

I am grateful to Mr. J. M. Hammersley of the Department for the Design and Analysis of Scientific Experiment, University of Oxford, for suggesting the above treatment of the problem. He derived the expressions for the effective areas of both core and periphery after making the following assumptions:

1. That egg fragments could be considered to be straight lines.
2. That these lines may lie in any direction; all directions, in three dimensions, being equally likely.
3. That the length of an egg fragment is independent of the direction in which it lies.
4. That all fragment lengths between zero and $2l$ are equally likely, l being the mean fragment length.

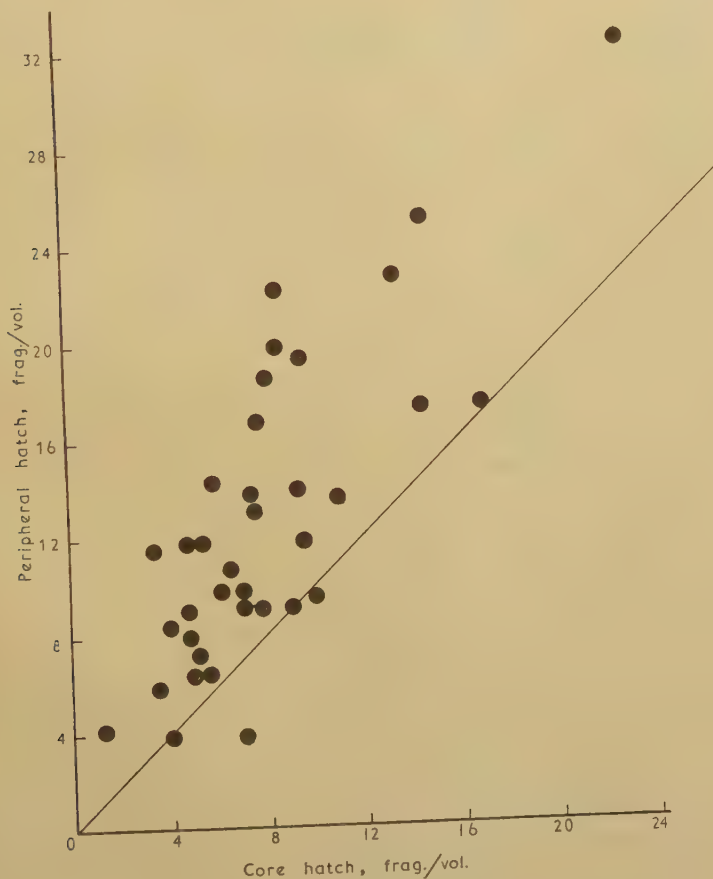


FIG. 6. Core hatch and peripheral hatch for cysts sectioned transversely.

The mean length of eggs from cysts used in the work was 95μ and a regression analysis showed that there was no significant tendency for egg length to vary with changes in cyst size ($p > 0.6$). The mean fragment length was

47.5 μ . The corrections derived from these values amounted to 18% of the core and 25–30% of the peripheral area.

RESULTS

1. Hatching from periphery and core

In fig. 6 values for the hatches from the periphery are plotted against corresponding values for the core of each of 36 cysts sectioned transversely, i.e.

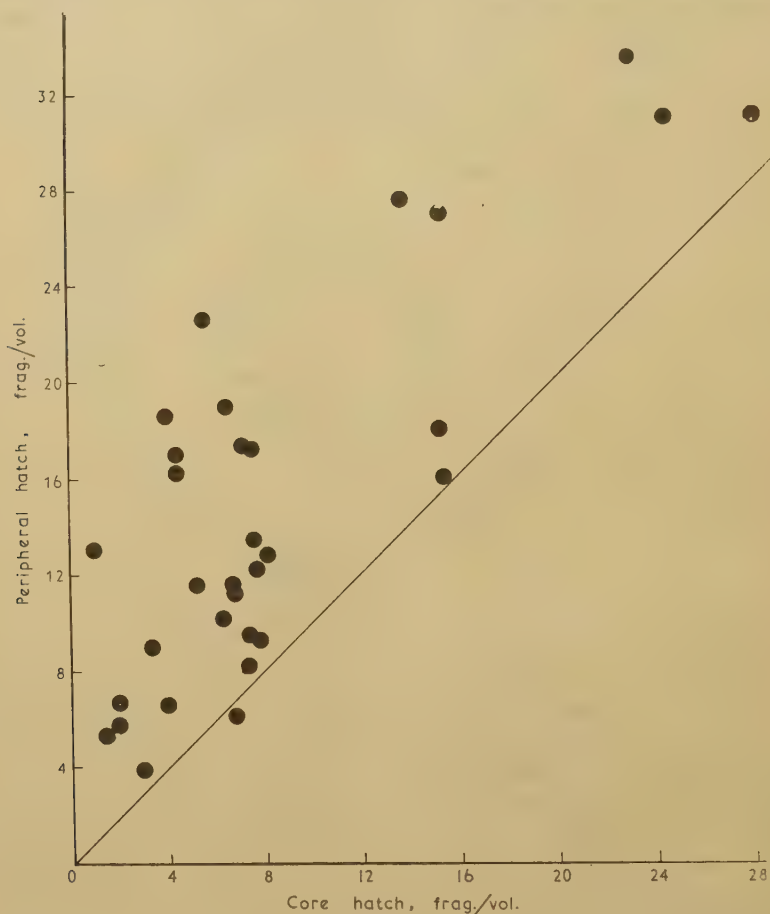


FIG. 7. Core hatch and peripheral hatch for cysts sectioned longitudinally.

with the plane of section at right angles to the longitudinal axis. Clearly, if the hatches in the two regions were equal, all values would lie on the line of slope 45° drawn in the diagram. In fact, no less than 32 of the 36 points lie above this line and the remaining four quite close to it. A similar diagram for 32 cysts sectioned longitudinally, i.e. parallel to the axis, is presented in fig. 7. In this case, 31 of the 32 points are above the 45° line. Clearly for both lots of cysts the peripheral hatch is greater than the core hatch in the majority of cases.

Comparison of the hatches by pairing, a test based on the differences between the core and peripheral hatches of single cysts, showed that in both cyst lots the differences between the hatches of core and periphery are very highly significant ($p < 0.001$ in both cases). There was considerable variation in the data; for transversely sectioned cysts, for example, the standard deviation for the differences between members of individual pairs was ± 3.96 f/vol. The results of the comparisons are shown in table 2a.

2. Hatching from the ends and periphery

In fig. 8 values for the hatches from the neck end are plotted against corresponding values for the peripheries of cysts sectioned transversely. The points

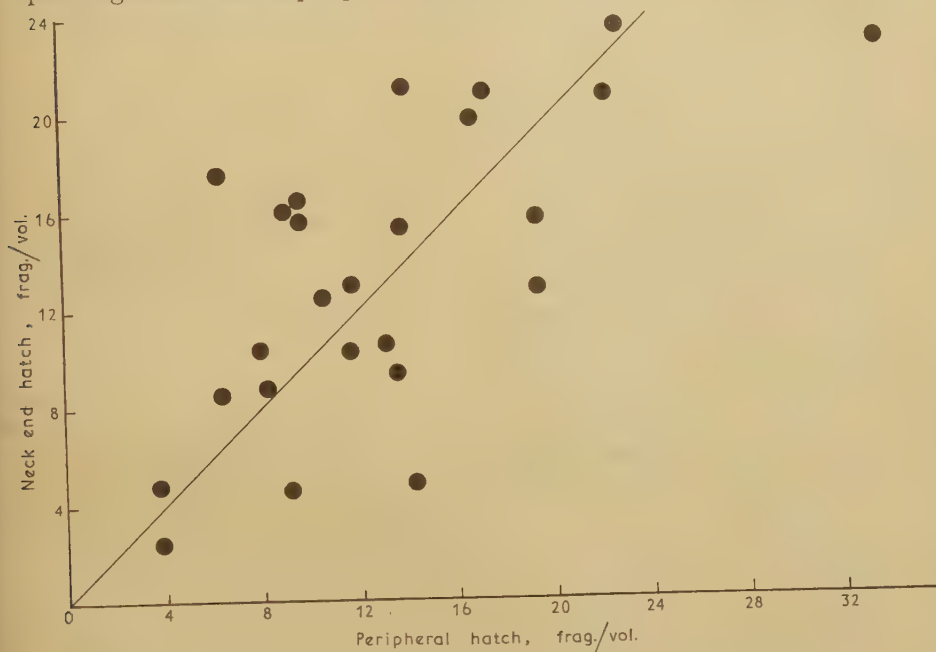


FIG. 8. Neck end and peripheral hatches for cysts sectioned transversely.

are fairly evenly distributed about the 45° line, i.e. the hatches from the two regions are very similar. Comparison by pairing shows that, in fact, there is no significant difference between them ($p > 0.6$). Fig. 9 is a similar diagram for the vulva end and peripheral hatches of the same cysts. Unlike fig. 8, fig. 9 shows clearly that, in general, the vulva end hatches are larger than the peripheral hatches, 15 of the points being above the 45° line and 7 below but fairly close to it. Comparison by pairing showed that the differences between the hatches of the vulva end and the periphery are, in fact, highly significant ($p < 0.01$).

In longitudinally sectioned cysts, the neck and, in the majority of cases, the vulva are incorporated in the central region, leaving the ends without apertures. Both ends were therefore considered to be alike and only one was

investigated. In fig. 10, values for the hatches from the end investigated are plotted against corresponding values for the peripheral hatches of 32 cysts sectioned longitudinally. Twenty-three of the 32 points lie above the 45° line, showing that on the whole the end hatches were greater than the peripheral

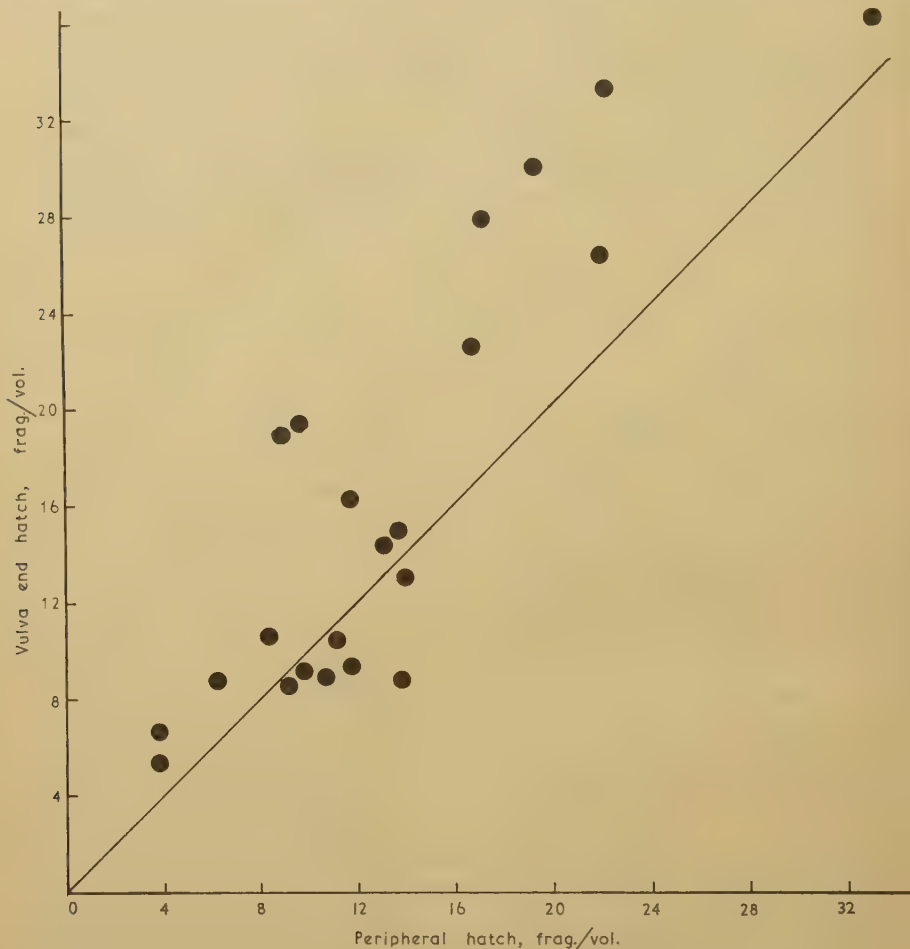


FIG. 9. Vulva end and peripheral hatches for cysts sectioned transversely.

hatches. Comparison by pairing confirmed that the differences between the end and the peripheral hatches are highly significant ($p < 0.01$). The results of all end-periphery comparisons are presented in table 2b.

Eelworm cysts may differ from one another in a variety of ways. For example, there may be genetical differences (Gemmell, 1940), differences between cysts from various potato varieties (Gemmell, 1943; Ellenby, 1946c), and differences in age, size, total emergence, and emergence per day, i.e. hatch rate. All cysts used were taken from potatoes of variety 'Redskin', and, although this is unlikely, the results may apply only to cysts of this variety.

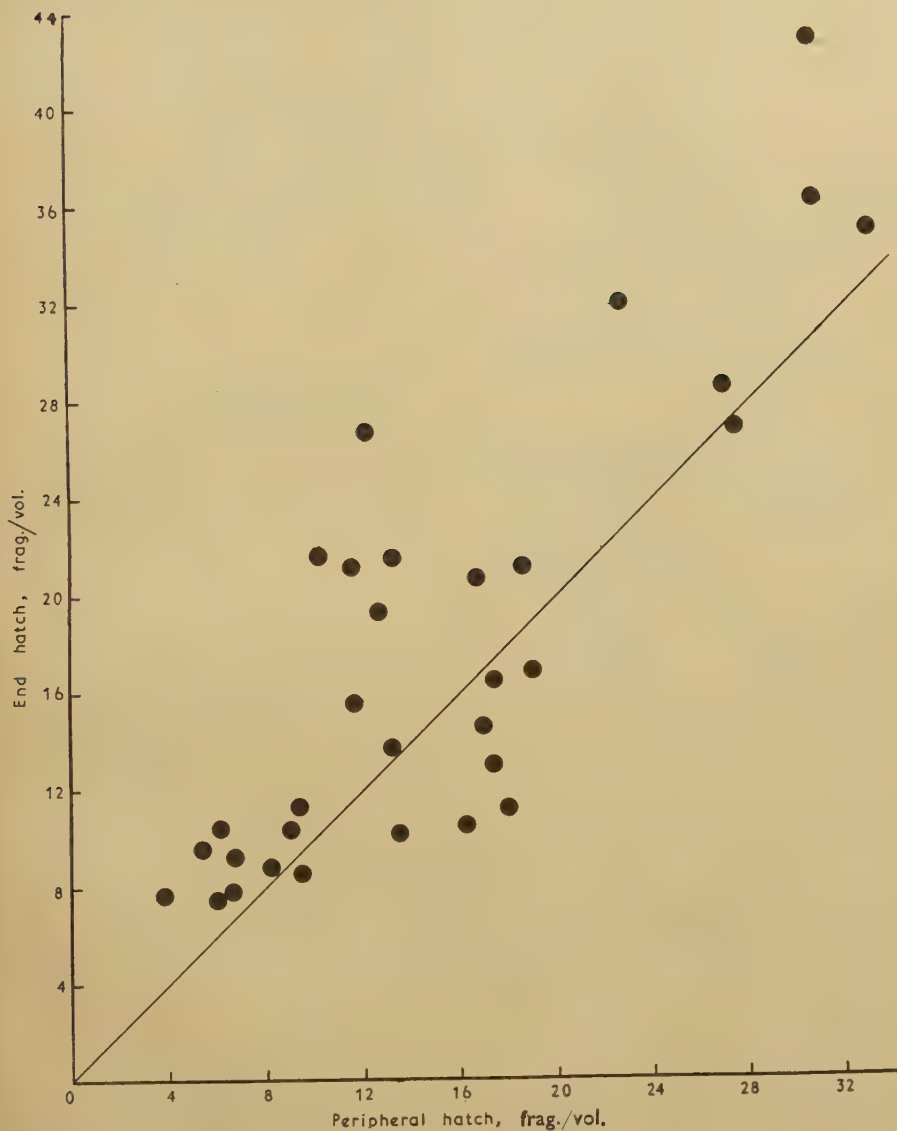


FIG. 10. End and peripheral hatches for cysts sectioned longitudinally (ends devoid of apertures).

TABLE 2

Differences between hatches from various regions of cysts sectioned either transversely or longitudinally

(a) Comparison of peripheral hatch and core hatch

	Mean value of ratio peripheral-hatch/core-hatch	Mean difference between peripheral hatch and core-hatch and S.E. (f/vol)	t	p
Cysts sectioned transversely	1.59/1	+4.75, ± 0.66	7.2	<0.001
Cysts sectioned longitudinally	1.77/1	+6.13, ± 0.84	7.3	<0.001

(b) Comparison of ends and peripheries

	Mean values of ratios	Mean difference between hatches and S.E. (f/vol)	t	p
Cysts sectioned transversely	Neck-end-hatch/ peripheral-hatch 1.03/1	+0.40, ± 1.00	0.4	>0.6
	Vulva-end-hatch/ peripheral-hatch 1.24/1	+3.04, ± 0.98	3.1	<0.01
Cysts sectioned longitudinally	End-hatch/ peripheral-hatch 1.19/1	+2.80, ± 0.90	3.1	<0.01

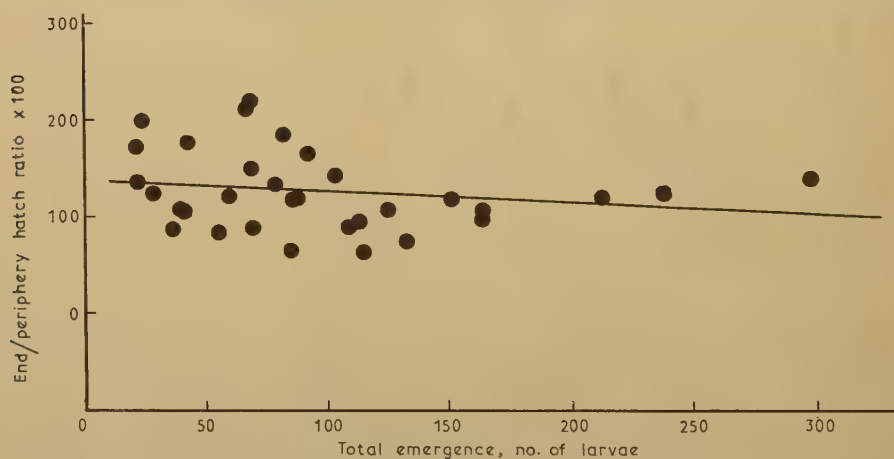


FIG. 11. End/periphery hatch ratio and total emergence for cysts sectioned longitudinally.

They were all more than two years old when the work began. As the other factors might well affect the hatching pattern, their influence was examined. In figs. 11, 12, and 13 the values of the ratio end-hatch/peripheral-hatch are

plotted against total emergence, hatch rate, and cyst size for cysts sectioned longitudinally. Regression analyses showed that total emergence and hatch rate had no significant effect on the ratio ($p > 0.2$ in both cases); however,

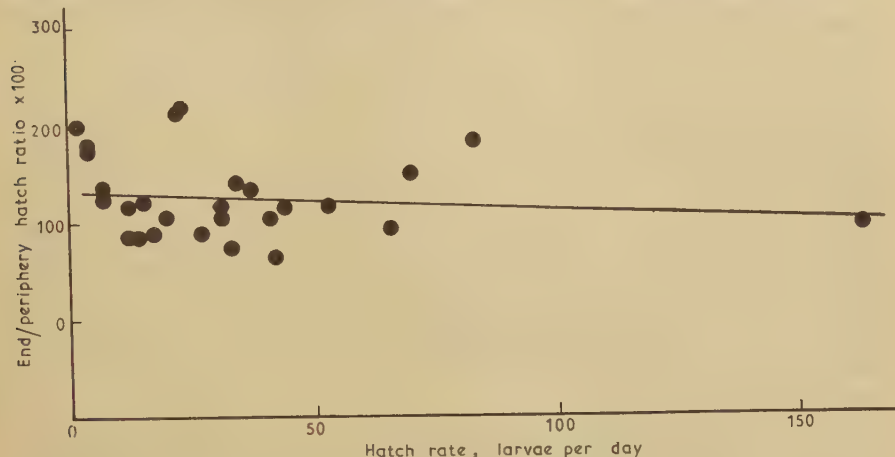


FIG. 12. End/periphery hatch ratio and hatch rate for cysts sectioned longitudinally.

there is a significant tendency for the ratio to increase as cyst size increases ($p < 0.01$).

The ratios peripheral-hatch/core-hatch, neck-end-hatch/peripheral-

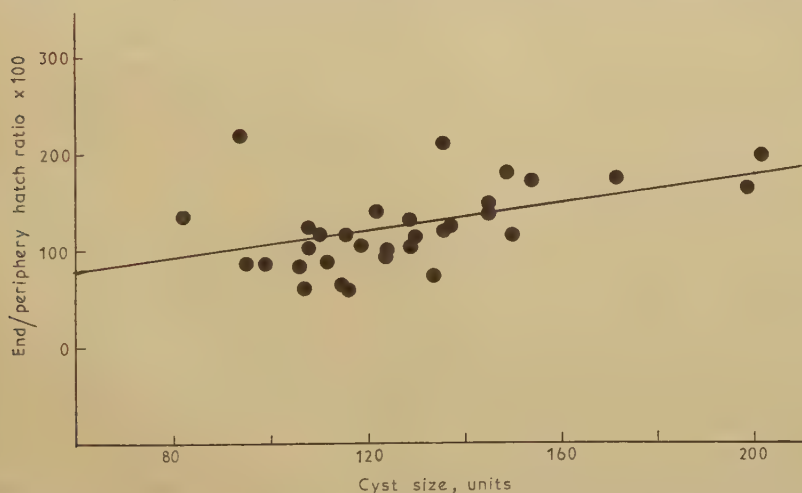


FIG. 13. End/periphery hatch ratio and cyst size for cysts sectioned longitudinally. With regression coefficient $+0.73$ and standard error of regression ± 0.24 , there is a significant tendency for the ratio to increase with cyst size ($p < 0.01$).

hatch, and vulva-end-hatch/peripheral-hatch for transversely sectioned cysts, and the peripheral-hatch/core-hatch ratio for longitudinally sectioned cysts were also tested. In no case did any of the three variables, total emergence, hatch rate, or cyst size have any significant effect.

DISCUSSION

The results show that hatching from cysts is not random. On the whole, eggs near to the cyst wall, i.e. in the periphery, tend to hatch first compared with eggs nearer the core. Apparently a 'hatching gradient' is set up.

Two factors are known to affect hatching; there may be others. Triffitt (1930*a*) showed that no hatching takes place in the absence of oxygen, and she also showed (1930*b*) that although a few larvae will emerge in water, large-scale hatching does not begin until the cysts are stimulated with root diffusate. Either of these factors might be capable of explaining the 'hatching gradient'. The possibility of root diffusate being the critical factor could be tested by examining the hatching pattern of cysts like those of the beet eelworm, from which there is some hatching in water alone, or by examining cysts from the field if the findings of Oostenbrink (1950) and Fenwick (1950) are of general validity. However, the balance of evidence already suggests that oxygen is the more important factor.

The cyst wall has been shown to be permeable to root diffusate (Ellenby, 1955); this might be thought to explain why the eggs near the wall hatch first. However, there is always some early hatching from the core even though it is shown to be less than from the periphery. Presumably, therefore, root diffusate reaches the core fairly quickly. Moreover, if root diffusate were responsible for the difference between periphery and core, the periphery-hatch/core-hatch ratio might be expected to decrease with time as hatching proceeds. In fact, as shown by figs. 8 and 9, neither total emergence nor hatch rate has any significant effect on the ratio. This suggests very strongly that some factor other than root diffusate is responsible for the hatching gradient.

The fact that cyst size does not affect the periphery/core ratio is at first rather puzzling, for when the core becomes farther from the wall the gradient should become steeper, i.e. the ratio should increase. However, as the size of the core is constant, increase in cyst size leads to an increase in the size of the periphery alone and therefore to the number of eggs contained in it. If hatching is reduced in the core because it is farther from the wall, it will also be reduced in the periphery because, as cyst size increases, there will be more and more eggs at a greater distance from the wall than there are in the peripheries of smaller cysts. The effects on the core and periphery may therefore balance and the ratio remain fairly constant with increasing cyst size. This, in fact, is found to be the case.

In the present work, total emergence had to be restricted to about 150 larvae per cyst because of technical difficulties associated with dealing with large numbers of fragments in the sections. Therefore in the majority of cases hatching was not allowed to proceed to the cessation of emergence. Thus it is by no means clear whether the factors bringing about the cessation of emergence are similar to the factors producing the gradient demonstrated in this work. But, clearly, root diffusate can have nothing to do with the cessation of emergence as this occurs even in the presence of freshly supplied root

diffusate; on the other hand, oxygen deficit could quite easily form part of the complex situation resulting in inhibition of hatching. And the present results are in agreement with the hypothesis that a gradient of oxygen tension is responsible for the hatching gradient.

Whether or not the gradient is one of oxygen tension, a gradient of some kind is clearly demonstrated. After stimulation there will be an increase in larval movement within the cyst; nevertheless the gradient exists in spite of this, indicating that the stirring effect caused by larval movement is negligible. This is not surprising, for the cysts are packed with eggs and egg shells and this baffles the effect of the movement.

A high oxygen tension at the outside of the egg mass would decrease towards the centre of the cyst even in the absence of any stimulation. Increase in larval activity could quickly steepen this gradient, helped possibly by proximity to the wall and therefore to the stimulus in addition to proximity to the oxygen. Hatching presumably ceases when the oxygen tension falls below a certain minimum level even in the presence of root diffusate. After stimulation, more oxygen may be required by the larvae, owing to their increased activity: if this oxygen is not replaced quickly enough, a deficit might develop causing inhibition of hatching. Further, the increased larval metabolic rate would result in increased production of CO_2 , which, although it might escape fairly quickly, could help to inhibit hatching by increasing the acidity of the medium. Ellenby (1946*b*) has shown that pre-treatment with acid solutions does cause reduction in larval emergence. During the interval between inhibition and re-commencement of hatching the oxygen deficit could be made good and the CO_2 concentration revert to normal.

The above hypothesis is in agreement with Ellenby's suggestion that inhibition occurs as a result of stimulation with root diffusate, i.e. as a result of increased larval metabolism within the confines of the eelworm cyst. The punctures in his cysts could speed the entry of oxygen and exit of CO_2 and thus delay the onset of inhibition. An hypothesis of oxygen deficit might also explain the difference in the levels of infection of heavy soils and light sandy soils. Triffitt (1930*a*) suggests that the higher degree of infection of the latter is due to its better aeration, i.e. to the availability of more oxygen. Further, Wallace (1954) suggests that high emergence from beet eelworm cysts in water is correlated with high oxygen concentration of the surface films.

The fact that, in general, the highest hatches are found in the ends of cysts is not inconsistent with the oxygen tension hypothesis. No egg contained in an end is ever more than 100μ from some part of the cyst wall; whatever the size of the cyst the eggs in the ends, taken as a group, would always be in a region of higher oxygen tension than those in the periphery. The end hatches would therefore tend to be higher. As cyst size increases, ends and peripheries both get bigger and their hatches would therefore, on hypothesis, decrease. But because of the relative distances from the wall of eggs in the ends and the peripheries, the end hatches would decrease more slowly and the end-hatch/periphery-hatch ratio might tend to increase. In fact, this has been shown to

be the case for cysts sectioned longitudinally (fig. 13, p. 509), in which the neck and vulva are incorporated in the central region.

Except as exits for the larvae, the part played by the natural apertures in hatching is not clear. The hatch from the neck end is not significantly greater than that from the periphery, but this may be due in some way to its abnormal shape. The end hatch has been shown to be significantly greater than the peripheral hatch and the mean value of the end-hatch/periphery-hatch ratio does not change significantly whether the vulva is included in the sections of the end or not. Both apertures are probably very small; accurate measurements are difficult but the apertures appear to be considerably smaller than the diameter of larvae. The apparent difficulty which the larvae have in emerging from either aperture under *in vitro* conditions supports this view. Eelworms are able to pass through holes considerably smaller than their own cross section; for example, the chrysanthemum eelworm, *Aphelenchoides ritzema-bosi*, enters leaves through the stomata (Stewart, 1921). The apertures of potato eelworm cysts are apparently unimportant in relation to the hatching pattern and the results of the present work are consistent with the view that root diffusate enters the cyst equally through all parts of the wall.

Little is known about what actually happens inside the cyst. The present paper gives some idea of the state of the cysts after varying periods of hatching. It enables some suggestions to be made about the causes of the distribution of hatching inside the cyst but much more evidence is needed before anything definite can be said on the matter.

I am grateful to Dr. C. Ellenby for his advice and encouragement at all stages of the work; also to Professor A. D. Hobson for his interest, and to all others who helped in any way. The work was financed by a grant from the Agricultural Research Council.

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Axial Illumination in Microscopy

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SUMMARY

A simple and accurate method of ensuring axial illumination is described. It involves examining a suitable cardboard disk held in the substage filter-holder and checking that the image of the field-stop is central in the field of view.

INTRODUCTION

AXIAL illumination is very important for critical microscopy and various methods have been proposed to ensure it, e.g. by Oettlé (1947) and Baker (1954). Oettlé's method, that of centring the reflexion from the substage filter with the light source, gives correct illumination provided that one applies the correction that is generally necessary to allow for the filter-holder not being set exactly at right angles to the optical axis. However, the reflected spot is often difficult to see without the inconvenience of darkening the room. Baker's mirror-cover is a very quick, simple method, but where a small-source Köhler system is used, such as the Pointolite, it may not be quite accurate enough, since there is an error of up to 3 mm, which depends on the distance from the actual reflecting surface of the mirror to the front surface of the mirror cover. It may be remarked that all these methods are unnecessarily delicate when a wide-angle light source is used.

METHOD

Axial illumination can be achieved very simply, quickly, and accurately in the following manner. First of all the microscope condenser is centred in the usual way, and also the iris diaphragm if this is independent. The lamp is set up at the correct distance from the microscope with its bulb properly centred to the field-stop. A circle of stiff white cardboard is then prepared of suitable size to fit into the substage filter-holder without movement. It is marked with an arrow so that it can be replaced in the same orientation; a convenient point for the arrow is the little knob on the filter-holder. If the mounting of the condenser and iris diaphragm can be removed without altering the centring, this is done and the condenser unscrewed from it. The iris diaphragm is closed to the diameter of a needle, which is passed through the iris as nearly at right angles to it as possible, to make a small hole in the disk, which is unlikely to coincide with its centre. With this hole as centre, draw a circle with a pair of compasses with a diameter equal to that of the lower lens of the condenser on the lower surface of the disk. The mounting of the condenser and iris is replaced without the disk and an object focused with the microscope; the field-stop is then focused with the condenser as usual. The lamp is roughly aimed

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and the image of the field-stop brought central with the field of view by movement of the mirror. Then the disk is replaced, circled side down, and the holder swung into position under the condenser. The disk is observed by means of a small mirror held in a simple holder of wood, cardboard, or metal. For convenience the mirror should be in such a position that the disk can be seen in it with the minimum of movement of the observer's eye from the eyepiece. The source of light is now focused on the disk by the bull's-eye of the lamp. The lamp is then pivoted until the image of the light source is symmetrical around the circle on the disk and completely covers it. The disk is swung aside, and the image of the field-stop is examined down the microscope. Owing to the fact that the axes of the pivots of the lamp do not pass through the field-stop, this will no longer be central. The image of the field-stop is centred by moving the mirror. The disk is replaced and the operation repeated. Two repetitions should be ample. When the image of the field-stop is central and the circle on the disk is filled with light, one can be sure of axial illumination and also of a completely filled condenser. Note that neither the filling of the condenser nor the centring of the image of the field-stop by itself ensures axial illumination, since the condenser can be filled by light almost at right angles to the axis, while the field-stop may be brought central by the mirror wherever the light may be directed.

During the whole process, neutral density-filters will be very necessary if a Pointolite is being used. Once axial illumination has been achieved, the disk is removed completely. If the filter-holder cannot readily be swung aside, it may be advantageous to cut segments out of the disk, giving it the form of a Maltese cross within a circle. This passes enough light to form an image of the field-stop, and so it may be left in position during the whole process of adjustment, but it must be removed before the microscope is actually used.

When written out the method sounds complicated; but once the disk has been made, the whole adjustment can be performed easily in 30 seconds.

It may be seen that this method possesses the following advantages:

It enables axial illumination to be achieved simply, quickly, and accurately.

It ensures that the condenser is filled with light.

The field lens on the lamp may be focused on the disk.

Only two very simple bits of apparatus are required.

It is not necessary for the filter-holder to be at right angles to the optical axis.

I should like to thank Dr. J. R. Baker for his advice and encouragement, and also the Medical Research Council, under whose grant I am working.

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Observations on the Stomach and Digestive Diverticula of the Lamellibranchia

I. The Anisomyaria and Eulamellibranchia

By G. OWEN

(From the Department of Zoology, University of Glasgow)

With 3 plates (figs. 1, 2, and 6)

SUMMARY

A study of the digestive diverticula of the Anisomyaria and Eulamellibranchia revealed certain features hitherto undescribed. The diverticula consist of blind-ending tubules which open into ciliated main ducts by way of short, non-ciliated secondary ducts. The main ducts open into the intestinal groove. In all the species examined the ciliated epithelium of the main ducts was restricted to a well-defined groove, the remainder of the lumen being surrounded by a non-ciliated, brush-border epithelium.

Each tubule is surrounded by a system of smooth muscle fibres. Cilia associated with the darkly staining cells of the crypts were demonstrated in sections of the tubules. After feeding with titanium dioxide in suspension, this substance was later found in the large vacuolated cells of the tubules. The particles of titanium dioxide were larger than 0.1μ .

A continuous circulation is maintained within the main ducts solely as a result of ciliary activity. The exhalant current in the ciliated portion of the main ducts produces an inhalant counterpart current in the non-ciliated portion. It is suggested that fresh fluid is drawn into the tubules as a consequence of the absorptive functions of the large vacuolated cells. Indigestible material accumulates in the large vacuolated cells and is extruded into the main ducts where it is conveyed out of the diverticula by the exhalant ciliary current.

In both the Anisomyaria and Eulamellibranchia the flap-like major typhlosole prevents material entering the mid-gut except by the intestinal groove, and isolates the rejectory currents of the intestinal groove from the general circulation of particles in the stomach. In the Eulamellibranchia the major typhlosole also acts as a valve which controls the entry and exit of material into and out of the inhalant and exhalant portions of the main ducts.

INTRODUCTION

MANY conflicting views have been expressed regarding the functions of the digestive diverticula of the Lamellibranchia. An absorptive function has been attributed to them by many authors, while others have regarded them as secretory organs (for details and bibliography see Yonge, 1926*a*). From a comparative study of the structure and functions of the diverticula of numerous lamellibranchs Yonge concluded that they were organs of absorption and intracellular digestion and this view has received general acceptance by the majority of workers. Recently, Mansour (1946 *a* and *b*, 1949) and Mansour-Bek (1946) have questioned the occurrence of intracellular digestion in the Lamellibranchia. They claim that extracellular proteases and lipases are secreted into the lumen of the stomach by the digestive diverticula.

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Despite this recent interest in the functions of the diverticula little has been added to our knowledge of the structure and mode of functioning of these organs.

Observations on the digestive diverticula of *Cardium edule* carried out during the course of feeding experiments revealed structural features hitherto undescribed, and these were subsequently investigated in other species of lamellibranchs. In this paper the results obtained for the Anisomyaria and Eulamellibranchia are described and their possible significance discussed.

The work was carried out on a variety of lamellibranchs with both fresh and fixed material. Fixed material was embedded in ester-wax (Steedman, 1947) and sections cut at $2-6\mu$ were in most cases stained in Heidenhain's iron haematoxylin, alcian blue 8GS (Steedman, 1950) and orange G in clove oil. Before embedding, the material was cleared in monochlorisothymol (Steedman, 1955).

THE STRUCTURE OF THE DIGESTIVE DIVERTICULA

The digestive diverticula surround the stomach and consist of numerous blind-ending tubules which have the form of globular (e.g. *Cardium*) or elongate (e.g. *Anodonta*) sacs or, as in the Mytilidae (List, 1902), of irregularly branched tubes with numerous saccular outgrowths. They communicate with the stomach by a system of ducts whose structure is distinct from that of the tubules.

Previous workers described the epithelium of the ducts as completely ciliated and resembling that of the stomach, but, as shown in figs. 1 and 2, A, B, and C, ciliated cells are restricted to a well-defined tract which frequently has the form of a groove or gutter, the cells round the remainder of the lumen being non-ciliated and possessing at their free margin a well-developed brush-border. This division of the epithelium of the ducts into two distinct regions was recognized by List (1902) in *Mytilus galloprovincialis*, but he failed to distinguish cilia from brush-border and so described the ducts as 'durchaus bewimpert, jedoch ungleichmäßig da das Epithel aus zwei vollkommen verschiedenen Elementen zusammengesetzt ist von denen jedes ungefähr die auf dem Querschnitt betrachtet auskleidet und jedem Abschnitt ein ganz besonderes Gepräge verleiht'. This division of the epithelium of the ducts is well shown in the Mytilidae and all the Anisomyaria examined. Dissections of the ducts of fresh specimens of *M. edulis* indicate that the ciliated groove is bounded on each side by a longitudinal ridge or typhlosole while the non-

FIG. 1 (plate). Sections of the ducts of the digestive diverticula of *Mytilus edulis*.

A and B, main ducts showing the division of the epithelium into ciliated and non-ciliated regions. A is a section of a main duct near its junction with the stomach. The typhlosoles are well developed while the brush-border of the non-ciliated epithelium is obscured by 'bubbling' of the epithelial surface. B is a section of the distal region of one of the main ducts. The typhlosoles are smaller than in A.

C, section of a secondary duct showing the well-developed brush-border and the absence of ciliated cells.

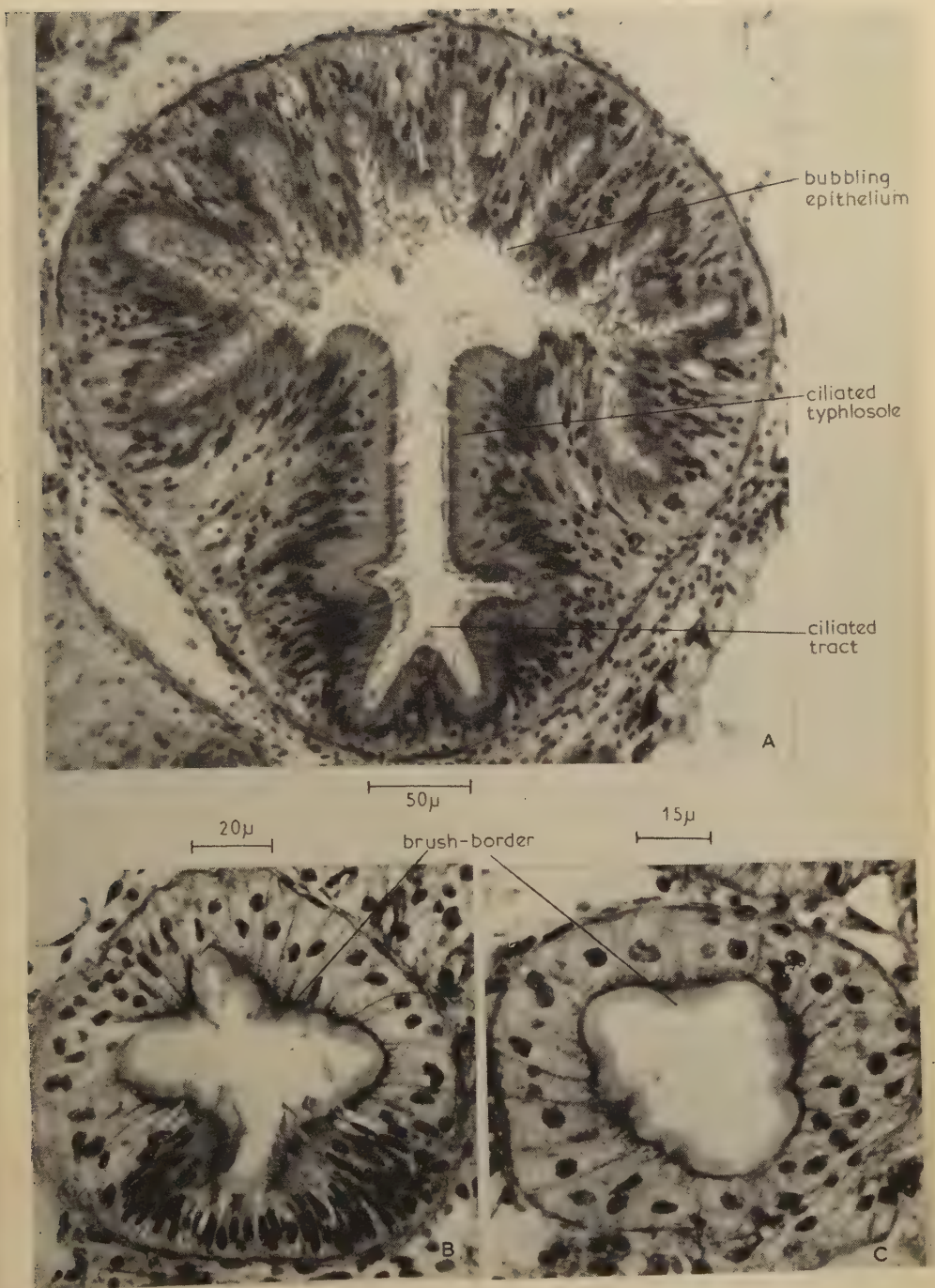
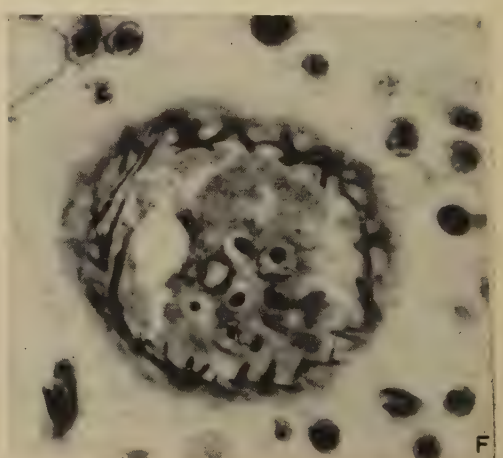
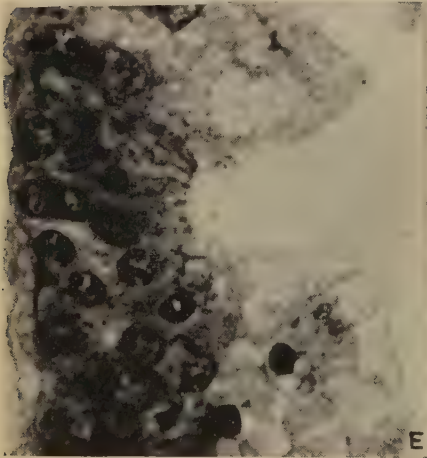
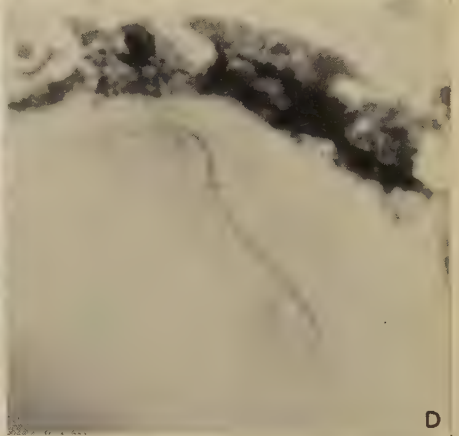
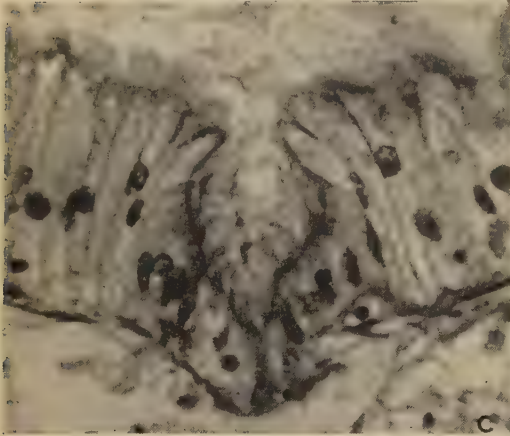
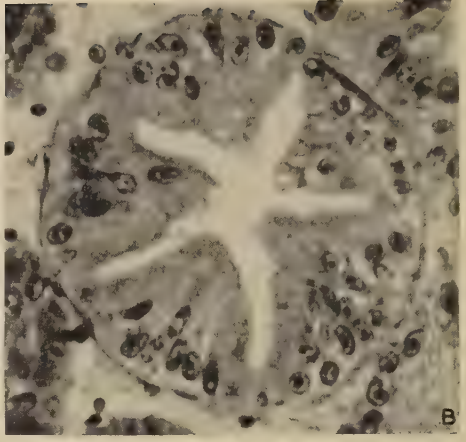


FIG. 1
G. OWEN



25μ
A-B

30μ
C

10μ
D-F

FIG. 2
G. OWEN

ciliated portion of the duct has a metallic sheen, possibly due to refraction from the surface of the brush-border epithelium. The typhlosoles partially divide the ducts into ciliated and non-ciliated regions, the division being most marked in the larger ducts near their junction with the stomach (compare fig. 1, A and B).

In *M. edulis*, the brush-border epithelium consists of cells of varying height containing spherical nuclei and possessing at the free margin a well-defined darkly staining region (fig. 1, B). In sections of fixed material the epithelium frequently includes numerous, apparently empty, vacuoles and the brush-border is often obscured by a characteristic 'bubbling' of the epithelial surface (fig. 1, A). The outline of the lumen of the non-ciliated portion is undulating owing to variation in height of the epithelium, and the bubbling cells are arranged in longitudinal bands coinciding with the taller epithelial cells. Knight-Jones (1953) described similar bubbling cells in the gut wall of *Saccoglossus* but he concluded that they were probably artifacts due to violent contraction on fixation. In contrast to the brush-border cells, the nuclei of the ciliated cells are elongate and only occasional vesicular outpushings are present at the epithelial surface (fig. 1, B). The typhlosoles are formed of tall ciliated cells, there being a gradual decrease in height of the epithelium toward the depth of the groove.

The ducts of the digestive diverticula of all the *Anisomyaria* examined are similar in cross-section to those of *Mytilus edulis* but members of the *Eulamellibranchia* exhibit variation in form both of the ciliated groove and of the brush-border epithelium. In *Spisula subtruncata* (fig. 2, c) the ciliated groove is well defined but small relative to the size of the ducts. Numerous mucous glands staining with alcian blue 8GS (Steedman, 1950) are present in the brush-border epithelium; they are not found in the majority of eulamellibranchs. In *Zirphaea crispata* (fig. 2, A) numerous villi project into the lumen of the ducts, the ciliated tract being situated between two villi. As in *Mytilus edulis*, however, dissections of these ducts show only two longitudinal typhlosoles, one on each side of the ciliated tract. In many *Eulamellibranchia* the ciliated groove is not as well defined as in the above species and the brush-border of the non-ciliated cells is frequently obscured by the bubbling appearance of the epithelial surface. Nevertheless, in all species of *Eulamellibranchia* examined ciliated cells were restricted to a well-defined tract.

FIG. 2 (plate). A, section of a main duct of the digestive diverticula of *Zirphaea crispata* showing the well-developed ciliated tract between two villi.

B, section of a secondary duct of *Zirphaea crispata*. Cilia are absent. Compare with A.

C, portion of main duct of the digestive diverticula of *Spisula subtruncata* showing the ciliated tract.

D, portion of a tubule of *Aloidis gibba* showing the cilia of the darkly staining cells.

E, portion of a tubule of *Ostrea edulis* showing the basal granules associated with the darkly staining cells. Staining of the cilia has been lost in order to differentiate the basal granules from the cytoplasm of the darkly staining cells.

F, section through the tip of a tubule of *Cardium edule* showing the network of smooth muscle fibres. See fig. 6.

The transition from the epithelium of the ducts to that of the tubules has been described as gradual in the Anisomyaria and as sharply defined in the Eulamellibranchia. A feature overlooked in previous descriptions of the digestive diverticula, however, is the existence in both the Anisomyaria and Eulamellibranchia of short, unbranched secondary ducts connecting the main ducts with the blind-ending tubules. These secondary ducts are non-ciliated, the cells being similar to those of the brush-border epithelium of the main ducts, but rarely is there any bubbling of the epithelial surface; the numerous vacuoles present in the brush-border epithelium of the main ducts are also

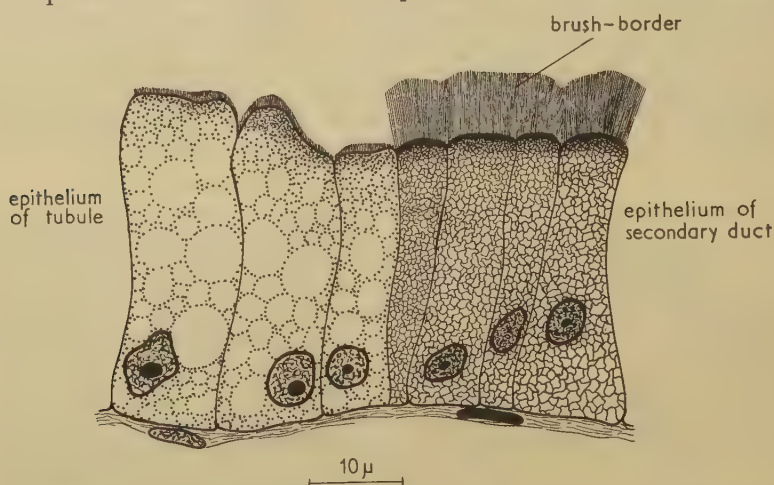


FIG. 3. *Mytilus edulis*, section showing the sharply defined junction of a secondary duct with a tubule.

absent (figs. 1, C and 2, B). Fig. 3 represents the junction between the epithelium of a secondary duct and of a tubule in *M. edulis*. The junction of the brush-border cells with the characteristically vacuolated cells of the tubules is sharply defined. Similar junctions were found in all the Anisomyaria examined. Thus, the duct system of the digestive diverticula is made up of main ducts and secondary ducts differing from one another both in their microscopic anatomy and their disposition relative to the tubules (fig. 4). The main ducts possess a ciliated tract. They leave the stomach and ramify through the mass of the diverticula but never communicate with the blind-ending tubules except by way of short, unbranched secondary ducts; each secondary duct leads into a cluster of tubules.

The structure of the tubules of the diverticula has been described by Yonge (1926a). Prior to this Potts (1923), as a result of observations on living material of *Teredo*, described the presence of cilia in both the normal tubules and in those specialized for the ingestion of wood fragments. He also described a ciliation similar to that of the normal tubules (these tubules are found exclusively in other lamellibranchs) in both *Xylophaga* and *Pholas*. Later, Yonge (1926a) observed cilia beating in the tubules of six other species of lamelli-

branches, but in most cases he was unable to determine whether they were present or not. Despite these clear indications of the presence of cilia in the tubules of the diverticula in fresh material, neither Potts nor Yonge was able to demonstrate them in sections of fixed material. Thus the cilia were described by Potts (1923) as 'easily retractile so that, when ordinary reagents are used for fixation of material it is impossible to demonstrate them in sections'.

During the present investigations, living material of as many species of Lamellibranchia as possible was examined. In addition to the three species

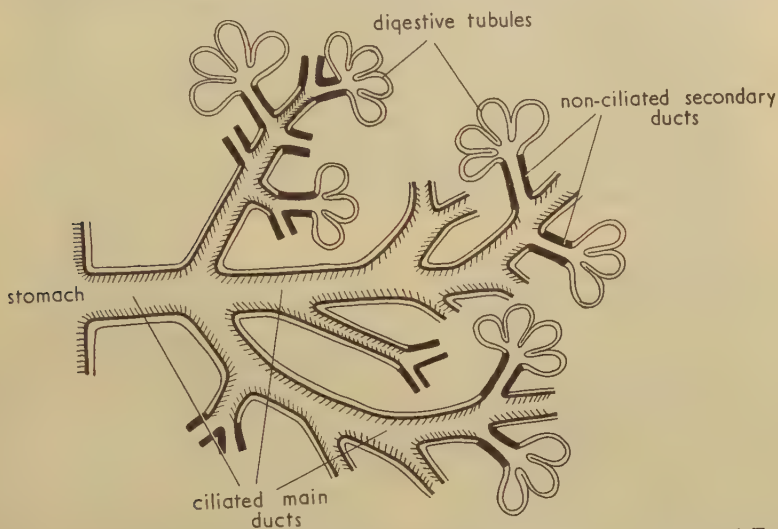


FIG. 4. The duct system of the digestive diverticula of the Anisomyaria and Eulamellibranchia shown diagrammatically.

in which Potts (1923) and the six species in which Yonge (1926a) observed cilia actively beating within the tubules, they were observed in *Venerupis pullastra*, *Cardium edule*, *C. echinatum*, *Glossus humanus*, *Arctica islandica*, *Venus fasciata*, *V. cassina*, *Astarte sulcata*, *Tellina tenuis*, *Abra alba*, *Cultellus pellucidus*, *Ensis siliqua*, *Aloidis gibba*, *Mya arenaria*, *M. truncata*, *Lutraria lutraria*, *Hiatella arctica*, *Zirphaea crispata*, and *Pholas dactylus*. In fact, cilia were observed in all the Eulamellibranchia examined. In species belonging to the Anisomyaria it was impossible from an examination of living material to determine whether cilia were present or not. Cilia were observed, however, in sections of fixed material in both the Anisomyaria and Eulamellibranchia. Fig. 2, D is a photomicrograph of the cilia of the tubules of *Aloidis gibba*.

The following description of the cells of the tubules is taken from Yonge (1926a):

The smaller darkly-staining cells are always present. They are found in small groups round the lumen in *Nucula* and all Filibranchs examined. They are often low, and lie between large vacuolated cells which meet above them and shut them off from the lumen. In the remaining lamellibranchs the lumen of the tubules is not

regular, as in the species just considered, but is elliptical, tripartite or in the form of a cross, with crypts (using the term employed by Gutheil, 1912) at the extremities of the two, three or four arms respectively in which lie the groups of dark cells.

In all the Eulamellibranchia and in *Ostrea edulis* in the Anisomyaria, these groups of darkly staining cells extend the length of the tubules to meet at the apex. Yonge concluded that they were nests of young cells which, by dividing,

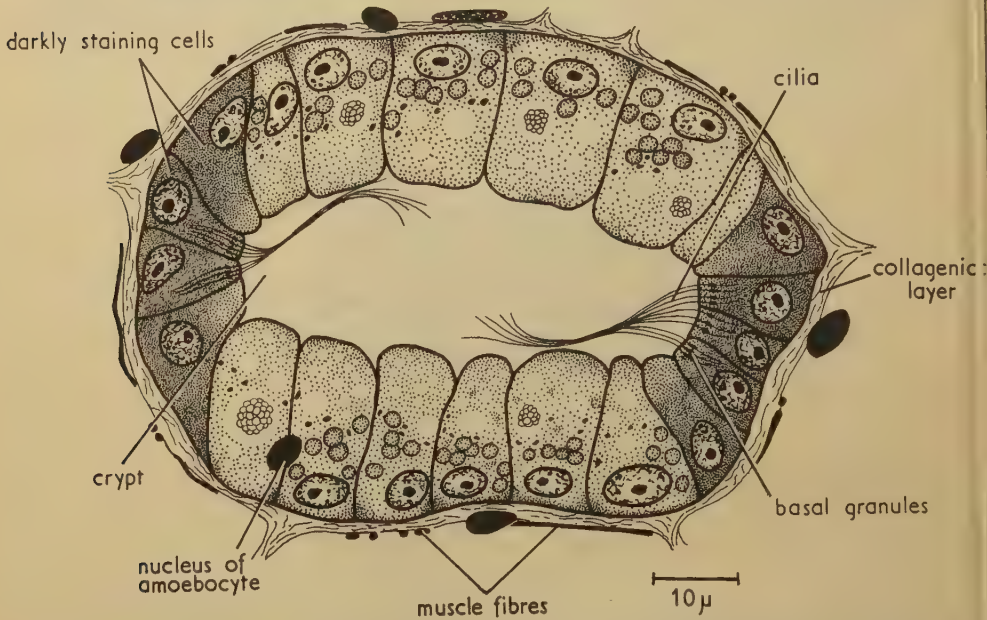


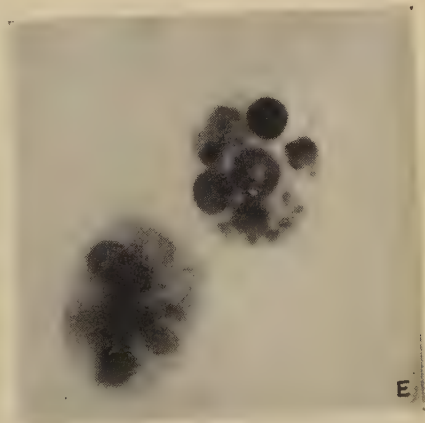
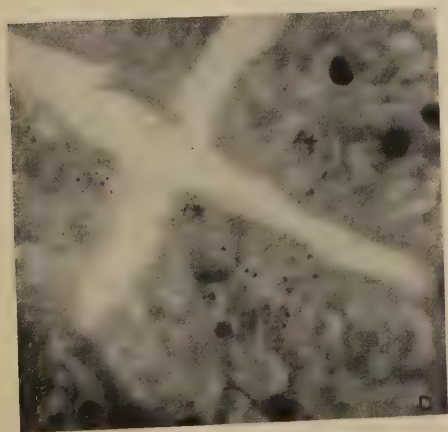
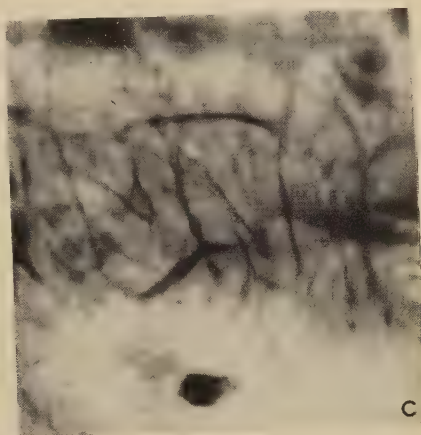
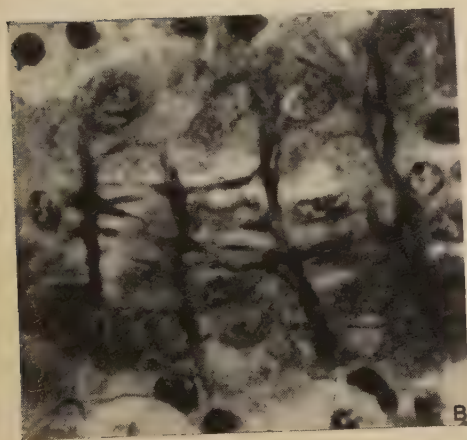
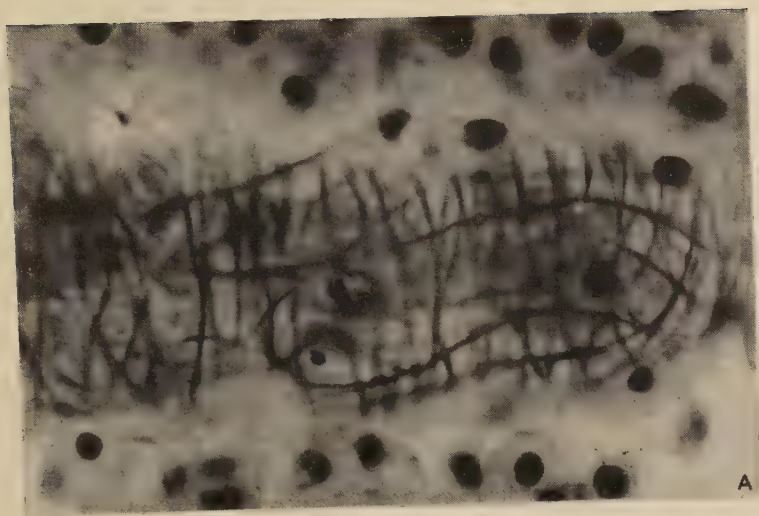
FIG. 5. *Venerupis pullastra*, transverse section through a tubule of the digestive diverticula showing the disposition of cilia and their association with the darkly staining cells of the crypts.

were able to make good the loss resulting from the casting off of the old cells. Mitotic figures were frequent in starved animals and always in the crypts. From an examination of sections of the tubules of numerous species there can be no doubt that the cilia of the tubules are associated with these darkly staining cells. It is the dark-staining property of these cells which makes the cilia so difficult to observe, but with careful differentiation it is possible to make out either the basal granules, or cilia, or both. Fig. 2, E is a photomicrograph of a portion of a tubule of *O. edulis*. The crypt has been cut transversely and a small number of basal granules can be seen near the free border of the

FIG. 6 (plate). A, B, and C are sections of the digestive diverticula showing the network of smooth muscle fibres surrounding the tubules in A, *Cardium edule*; B, *Ostrea edulis*; C, *Aloidis gibba*.

D, section of a tubule of *Ostrea edulis* showing particles of titanium dioxide which have been ingested by the cells of the tubules.

E, 'excretory' spheres of *Cardium edule* containing spherules packed with colloidal graphite.



10 μ
A-C

20 μ
D

10 μ
E

FIG. 6
G. OWEN

darkly staining cells. Staining of the cilia has been lost in order to differentiate the basal granules from the dark-staining cytoplasm, but the section demonstrates the association of the cilia with the darkly staining cells of the tubules.

A section through a single tubule of *Venerupis pullastra* is shown in fig. 5. In this species the tubules are elliptical with a group of dark cells at each of the extremities of the long axis. Long, fine cilia extend into the lumen of the tubule while the basal granules can be seen near the free border of the darkly staining cells. It is extremely difficult to determine the length of the cilia accurately since the entire length of a cilium rarely lies in the plane of a single section, but in *V. pullastra* and *Aloidis gibba* they are at least 30μ long. In most species they are probably somewhat shorter.

In addition to the above observations it was discovered that a system of smooth muscle fibres surrounds each tubule—a feature which although well known in the Crustacea does not appear to have been previously described in the Lamellibranchia. Portions of the diverticula were fixed in Bouin's fluid and in modified Bouin-Dubosq (Atkins, 1937), and sections cut at $4-6\mu$ were stained by one of the following methods: Heidenhain's iron haematoxylin, Heidenhain's Azan, Gurr's (1953) trichrome stain for elastin, smooth muscle, and collagenic fibres, and Linder's (1949) method for collagen and reticulin. The results indicated the presence of a variously developed skeletal sheath of collagen round each tubule immediately beneath the basement membrane (fig. 5); this layer of collagen is probably comparable with the *tunica propria* round the tubules of the digestive gland of Crustacea. Surrounding the skeletal sheath and partially embedded in it is a system of smooth muscle fibres forming a meshwork rather like a string bag. This 'string bag' effect is well shown in photographs of the fibres of *Cardium edule* (figs. 2, F and 6, A), *Aloidis gibba* (fig. 6, c), and *Ostrea edulis* (fig. 6, B), and these serve better than any description to illustrate their arrangement. Similar fibres, differing only in degree of development, were present in all species examined. As will be seen from fig. 6, A, the fibres are arranged circularly and longitudinally although it may well be that they have a helicoidal arrangement, the circular fibres being wound in a close spiral while the longitudinal fibres form an open spiral. It is interesting to note that the circular fibres in *O. edulis* (fig. 6, B), unlike the other two species figured, are better developed than the longitudinal fibres.

FUNCTIONING OF THE STOMACH AND DIGESTIVE DIVERTICULA

Most workers are agreed that digestion in the Lamellibranchia is a combination of intracellular and extracellular processes. The crystalline style projecting from the style sac into the stomach is the source of extracellular carbohydrases (Yonge, 1926b), and recent work by Mansour-Bek (1948) and George (1952) suggests that small amounts of extracellular proteases and lipases are also present although their source is uncertain. The tubules of the digestive diverticula are concerned with the absorption of soluble matter and the ingestion and intracellular digestion of fine particles; larger particles are ingested and

digested by amoebocytes (Yonge, 1926 *a* and *b*). Thus, fluid together with fine particles must be conveyed from the stomach to the digestive diverticula while waste material, consisting of rejected particles and excretory products, must be conveyed in the opposite direction, i.e. from the diverticula to the stomach. The role of the digestive diverticula as organs of intracellular digestion has recently been questioned (Mansour, 1949). Support for the view of intracellular digestion is based largely on the results of feeding experiments with Indian ink, carmine (List, 1902; Vonk, 1924), and iron saccharate (Yonge, 1926*a*). Krijgsman (1928) and G. and S. Hörstadius (1940) claim that these substances contain particles small enough to pass through the cells by some physical means and that phagocytosis, as suggested by Hirsch (1925), should be defined as a process of ingesting particles larger than 0.1μ . To test the phagocytic properties of the digestive diverticula Zaki (1951) fed animals with powdered gold-fibrin. No free gold particles were found in the cells of the diverticula and this was taken to indicate a lack of phagocytic properties on the part of these cells. Both Mansour and Zaki (1946) claim that the digestive diverticula are organs of secretion. There can be no doubt, however, from feeding experiments with iron saccharate, that the digestive diverticula are certainly organs of absorption and that fluid at least must be conveyed from the stomach to the diverticula.

To determine whether fine particles are also conveyed from the stomach to the diverticula, there to undergo phagocytosis, animals were fed with a suspension of titanium dioxide which was specially prepared by Acheson Colloids Ltd. It contained *no particles below* 0.5μ , the majority being about 1.0μ with occasional particles up to 2.0μ . Animals were placed in suspensions of titanium dioxide in filtered sea-water and fixed after definite periods. Before fixation, fresh preparations of the diverticula were examined under the microscope. Four species were used—*Ostrea edulis*, *Venerupis pullastra*, *Cardium edule*, and *Tellina tenuis*—and in each case free particles of titanium dioxide were present in the lumen of the tubules. From an examination of fresh material it was impossible to tell whether any particles were present within the cells or not. In sections of fixed material, however, particles of titanium dioxide, showing black by transmitted light and a characteristic white by reflected light, were easily identified within the large vacuolated cells of the tubules. Fig. 6, D shows a section of the diverticula of *Ostrea edulis* fixed 3 hours after feeding with titanium dioxide. It is hoped to discuss further details of this experiment elsewhere, but in so far as it concerns the problem of the functioning of the diverticula it demonstrates convincingly the passage of material from the stomach into the diverticula and the ability of the cells of the tubules to ingest particles larger than 0.1μ in size. Thus a two-way circulation must be maintained within the diverticula, fluid and solid particles being conveyed from the stomach to the tubules while waste materials, with or without secretions, are conveyed in the opposite direction. Previous workers (Yonge, 1937) have suggested that cilia maintain a continuous circulation within the diverticula, while recently Graham (1949), Owen (1953), and

Purchon (1955) have all suggested that the part played by muscular activity in the functioning of the gut of lamellibranchs, and in particular of the stomach and digestive diverticula, has not been fully appreciated.

Muscular activity

In the Crustacea the tubules of the digestive gland are surrounded by a network of striated muscles, the passage of fluid from and into the diverticula being controlled by the pumping action of the walls of the tubules (Pump, 1914). Morse (1902) described similar pumping movements of the 'stomachal glands' of brachiopods, and sections of *Terebratulina retusa* show a system of muscle fibres round the tubules similar to that found in the Lamellibranchia (personal observation). Thus changes in the contents of the diverticula of lamellibranchs could be brought about by the contraction and relaxation of the muscle fibres surrounding the tubules. But pulsations of the tubules were never observed in living specimens and there is no direct evidence, therefore, that in the Lamellibranchia these muscle fibres effect changes in volume of the tubules.

Patterson (1933), working on the comparative physiology of the gastric hunger mechanism, introduced a rubber balloon into the stomach of the large gaper clam, *Schizothoerus nuttallii*, and so recorded pressure changes there. Slight stomach contractions occurred at the rate of one per minute regardless of the presence or absence of the crystalline style; the contractions also continued in the absence of foot movements. Changes in pressure brought about by contractions of the stomach would aid the entry of material into the digestive diverticula and an attempt was made to repeat Patterson's experiment with *Cardium edule* and a different recording apparatus. Slight gastric contractions were recorded but it was impossible to determine whether these resulted solely from the irritation of the stomach epithelium or not. Direct observations of the stomachs of dissected animals did not reveal any rhythmic contractions and thus there is little to suggest that muscular contractions play an important part in the circulation of material within the diverticula. In the larval oyster, particles are drawn into the diverticula and returned to the stomach by the rhythmic expansion and contraction of the diverticula. But these are simple and sac-like in the oyster larva and unlike the much divided structure of the adult (Millar, 1955). Complicated and much-branched diverticula are present in the majority of adult lamellibranchs, and it is unlikely that rhythmic contractions similar to those which occur in the larva would result in an efficient circulation of material. Also, it is difficult to visualize such contractions taking place in species in which the digestive diverticula are closely invested by the surrounding connective tissue, e.g. *Mytilus edulis* and *Ostrea edulis*.

Ciliary activity

Three species, *Mytilus edulis* (figs. 1, A and B), *Ostrea edulis*, and *Zirphaea crispata* (fig. 2, A), provided suitable material for determining the pattern of

ciliary activity within the main ducts of the digestive diverticula. In all three species the cilia in the depth of the groove beat along the length of the ducts and away from the tubules. The cilia on the lower sides of the typhlosoles beat into the groove while those on the upper sides of the typhlosoles beat obliquely out of the groove (fig. 7). *There are no cilia beating from the stomach towards the tubules.* Although this pattern of ciliary activity was confirmed in only three species, it is probable that similar currents are present in all Anisomyaria and Eulamellibranchia where the cilia of the main ducts are restricted to a well-defined tract.

The cilia of the tubules were observed in fresh preparations of the diverti-

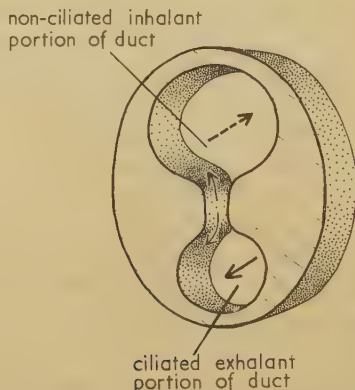


FIG. 7. A diagrammatic representation of a main duct of the digestive diverticula. Unbroken arrows indicate the direction of ciliary currents in the exhalant portion of the duct and on the typhlosoles, while the broken arrow represents the non-ciliary, inhalant, counterpart current.

cula of numerous species of Eulamellibranchia and are interesting in that they beat with an undulating or sinusoidal movement rather than a simple flexing or bending motion; they do not exhibit metachronal rhythm nor do they appear to produce any directive currents. Particles present in the lumen of the tubules appear to move solely as a result of direct contact with these cilia.

The cilia of the main ducts and of the tubules represent the entire ciliation of the digestive diverticula, and although there are no cilia in the main ducts beating towards the blind-ending tubules it is possible that a two-way circulation is maintained within these ducts solely as a result of ciliary activity. In any closed fluid system, as represented by the digestive diverticula, a ciliary current in the one direction inevitably carries a counterpart current in the opposite direction elsewhere. As a consequence of this, material is conveyed out of the diverticula in the ciliated portion of the main ducts while an inhalant counterpart current carries material in the opposite direction in the non-ciliated portion. The cilia of the typhlosoles, beating into and out of the ciliated groove, keep the two currents distinct. Fig. 7 is a diagrammatic representation of this two-way flow within the main ducts. Although there is morphologically only a single duct it serves functionally as two ducts, the ciliated portion carrying an exhalant current out of the diverticula and the non-ciliated portion an inhalant

current in the opposite direction. In this way a continuous circulation could be maintained within the main ducts solely as a result of ciliary activity. This circulation does not, however, include the lumen of the blind-ending tubules since between them and the main ducts are the short, non-ciliated secondary ducts (fig. 4). Interchange of material between the tubules and the main ducts must result from some means other than ciliary activity.

A feature which has been overlooked in considering the entry of fluid and particles into the lumen of the tubules is the absorptive function of the large vacuolated cells. That these cells possess an absorptive function has been

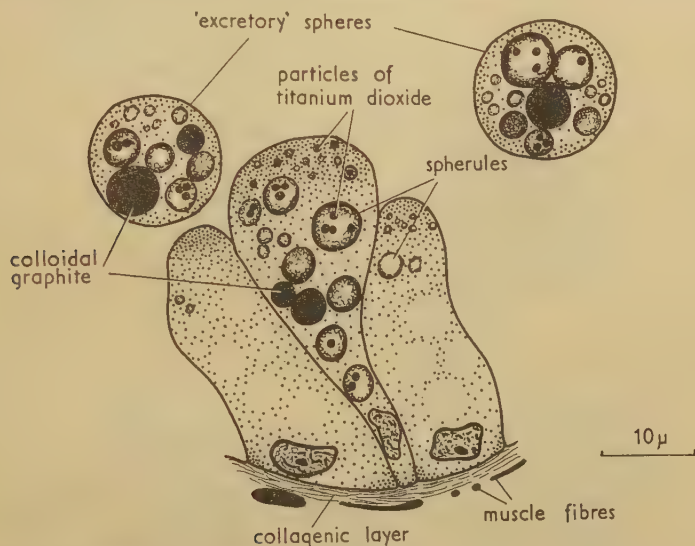


FIG. 8. Cells of a tubule of the digestive diverticula of *Cardium edule* 6 hours after feeding with a mixture of titanium dioxide and colloidal graphite.

demonstrated by various workers using carmine, Indian ink (List, 1902; Vonk, 1924), and iron saccharate (Yonge, 1926a). The short, unbranched secondary ducts open into the non-ciliated portion of the main ducts, and thus absorption by the cells of the tubules must inevitably result in fresh fluid being drawn into the lumen of the tubules from the inhalant current of the main ducts. Such a mechanism explains why only very fine particles are found in the tubules, since only those remaining in suspension in the fluid medium can enter in this way; *the rate of absorption controls the size of particle available*.

A few cells of a tubule of *Cardium edule* 6 hours after feeding with a mixture of titanium dioxide and Aquadag (colloidal graphite) are represented in fig. 8. Similar experiments producing identical results have been described by List (1902), Vonk (1924), and Yonge (1926a). Only an abbreviated account is therefore presented here. Both Aquadag and titanium dioxide have been ingested by the cells and are concentrated in spherules 3–5 μ in diameter. Some of the spherules contain titanium dioxide particles together with relatively small amounts of Aquadag scattered round the periphery while others are a solid

black mass of Aquadag so that it is impossible to tell whether they contain particles of titanium dioxide or not. This concentration of ingested particles into spherules is also indicated in the photomicrograph of a tubule of *Ostrea edulis* which had been fed with titanium dioxide (fig. 6, D). Present in the lumen of the tubules and of the ducts of *Cardium edule* were numerous large spheres, 12–18 μ in diameter, enclosing a varying number of spherules containing Aquadag and titanium dioxide and having an appearance similar to those within the cells of the tubules (figs. 6, E and 8). They are produced by the

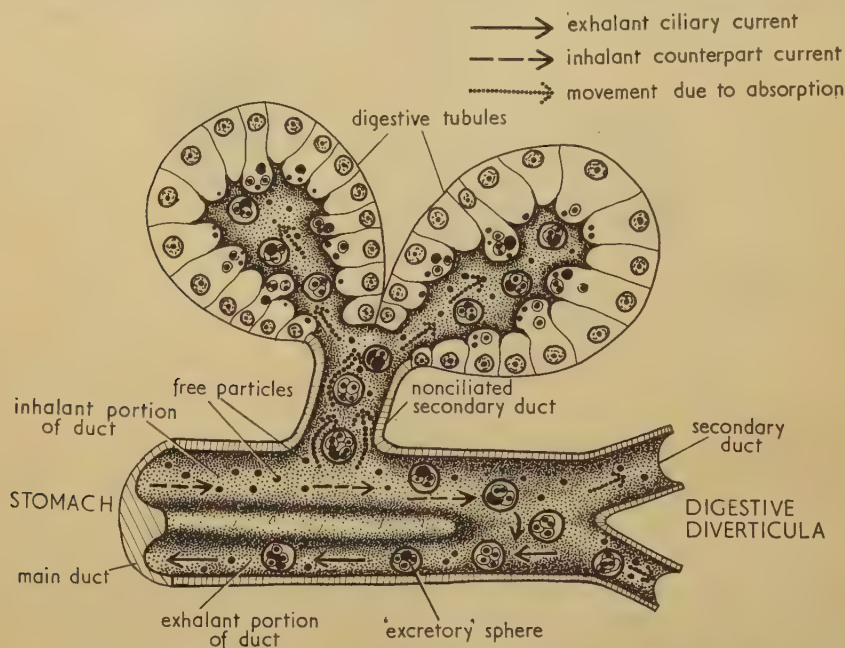


FIG. 9. The probable circulation of fluid and particles within the digestive diverticula of the Anisomyaria and Eulamellibranchia shown diagrammatically. Unbroken arrows represent ciliary currents; broken arrows a non-ciliary, inhalant, counterpart current; dotted arrows, movement due to the absorption of fluid by the cells of the tubules.

fragmentation of the tubule cells and serve to convey indigestible material out of the diverticula.

How the spheres leave the tubules is not certain. The continual fragmentation of the cells of the tubules could result in previously formed spheres being pushed through the short secondary ducts and so into the main ducts. Owing to their spherical form the spheres would not prevent fresh fluid continuing to enter the tubules. Alternatively, the tubules could be emptied by muscular activity. Dr. C. F. A. Pantin, F.R.S. (personal communication), has suggested that 'there are strong mechanical reasons for considering that the circular and longitudinal fibre systems [surrounding the tubules] have distinct functions. Circular systems of this sort [fig. 6, A] must inevitably be partly, if not wholly, concerned with tonic balancing of internal pressure while the

longitudinal fibres have necessarily much less to do with this and, in species in which they are well developed [e.g. *Cardium edule*], their prime function may be the emptying of the tubules.' Thus, it could be that the longitudinal fibres, from time to time, contract slowly to empty the contents of the tubules into the main ducts; the circular fibres, on the other hand, merely balance the weak pressure resulting from the absorptive functions of the cells.

In fig. 9 an attempt has been made to represent diagrammatically the suggested circulation of fluid and particles within the digestive diverticula. A continuous circulation is maintained within the main ducts solely as a result of ciliary activity, the inhalant counterpart current together with suspended particles being carried in the non-ciliated portion of the duct. As a consequence of the absorptive activities of the large vacuolated cells, fluid enters the lumen of the tubules by way of the short, non-ciliated secondary ducts. Soluble matter is absorbed and fine particles ingested. Indigestible material and waste products are concentrated in spherules and returned to the main ducts within large 'excretory' spheres. These excretory spheres are conveyed out of the digestive diverticula by the exhalant ciliary currents. The transfer of material from the inhalant to the exhalant portion probably occurs in the distal regions of the main ducts since here the ciliated typhlosoles which separate the two portions are not as well developed as in the proximal regions (compare fig. 1, A and B). They finally terminate near the distal ends of the main ducts (fig. 9). Thus the main ducts can be regarded as tubes bent on themselves to form U-shaped structures within which a continuous circulation is maintained. The spheres are unable to re-enter the tubules from the main ducts since they are almost certainly too large to be affected by the relatively weak absorptive flow. In this way, waste material is separated from the inhalant 'food' particles and soluble matter.

The stomach

It is now possible to discuss the function of some of the more obvious morphological features of the stomach. One of the most constant features of the stomach of the *Anisomyaria* and *Eulamellibranchia* is the prominent flap-like major typhlosole which extends from the combined style sac and mid-gut or, if they are separate, from the mid-gut anteriorly across the floor of the stomach to enter the caecum or its representative (Graham, 1949). In discussing the lamellibranch stomach Graham points out that 'elaborate mechanisms are necessary to ensure the movement of food and waste into and out of the ducts of the diverticula, where absorption and digestion of food particles occur. This is provided by the complex course and arrangement of the major typhlosole.' The importance of the major typhlosole in isolating the rejectory currents of the intestinal groove from the main stomach cavity has already been emphasized (Owen, 1953) but how the major typhlosole ensures the entry of material into the diverticula has not been described.

A detailed account of the ciliary currents of the stomach of *Zirphaea crispata* has been given by Purchon (1955) and a similar account for the stomach of

Glossus humanus by Owen (1953). The ciliary currents of the stomach of *Cardium edule*, apart from minor differences, are similar to those of *Glossus humanus*. Particles entering the stomach from the oesophagus are caught up by the revolving crystalline style and brushed on to the ridged surface of the posterior sorting area. Coarse particles are directed to the mid-gut by way of the rejection groove and intestinal groove while fine particles are directed first dorsally into the dorsal hood and then ventrally over the posterior wall of the stomach. Finally, they are caught up by the strong anteriorly directed currents of the major typhlosole and conveyed towards the openings of the right and

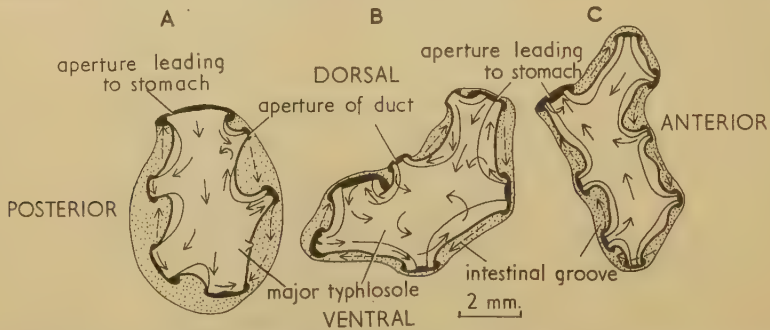


FIG. 10. The right caeca of A, *Glossus humanus*; B, *Cardium edule*; C, *Zirphaea crispata*. Unbroken arrows indicate the course of ciliary currents over the major typhlosole and broken arrows the rejectory currents of the intestinal groove.

left caeca. In all three species, particles failing to enter the caeca continue anteriorly to rejoin material entering the stomach from the oesophagus and so circulate again (fig. 13).

In the caeca of *G. humanus*, the ciliary currents over the extension of the major typhlosole are directed from the stomach to the apertures of the ducts of the diverticula (fig. 10, A). At the entrance to each duct, however, the ciliary currents are directed at right angles to the long axis of the duct. They do not convey particles directly into the ducts. In *Cardium edule* the pattern of ciliary activity in this region is more confusing (fig. 10, B). While the general trend of the currents is towards the apertures of the ducts there are a number of currents directed away from the ducts. This results in the formation of vortices where, at least in dissected specimens, particles entering the caecum from the stomach tend to collect; they do not enter the ducts. In *Zirphaea crispata* the pattern of ciliary activity is again a simple one, but, unlike *Glossus humanus*, the currents are all directed away from the openings of the ducts and towards the stomach (fig. 10, C). Thus in all three species ciliary currents do not convey particles directly into the ducts of the diverticula.

In the Eulamellibranchia the form of the major typhlosole within the caeca is that of a tube within a tube. Fig. 11 is a diagrammatic representation of the eulamellibranch caecum of species such as *Cardium*, *Mya*, and *Zirphaea*. The incomplete inner tube, formed by the major typhlosole, is continuous with the stomach cavity, while the outer tube formed by the wall of the caecum consti-

tutes the intestinal groove and is therefore continuous with the mid-gut. Extensions of the typhlosole project a short distance into the non-ciliated portions of each of the main ducts, while the ciliated portion opens on to the intestinal groove. As a consequence of this disposition of the major typhlosole within the caecum, waste material passing out of the diverticula in the ciliated portions of the main ducts is caught up by the rejectory currents of the intestinal groove, so preventing its return to the circulation within the stomach: fluid and fine particles conveyed from the stomach to the proximity of the ducts by the ciliary currents of the major typhlosole are drawn into the non-

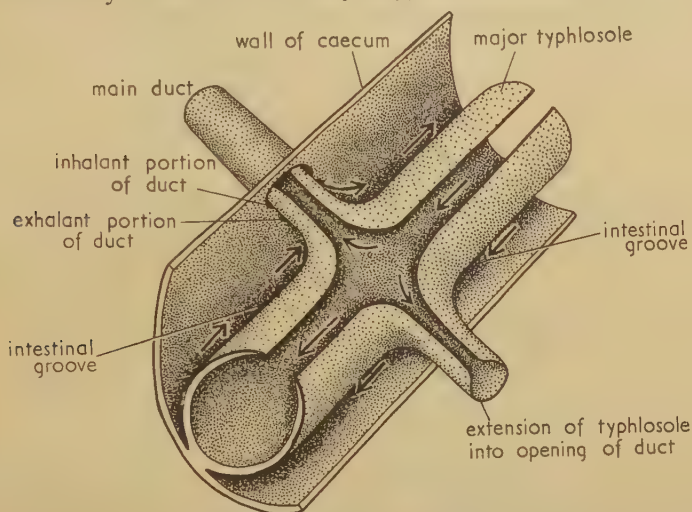


FIG. 11. A diagrammatic representation of the form of the major typhlosole within the caecum of most eulamellibranchs. Ciliary currents are indicated by arrows. The direction of those over the major typhlosole will vary in the different species (see fig. 10).

ciliated portions of the main ducts by the inhalant counterpart current. Thus the extension of the typhlosole into the caecum acts as a valve, allowing fluid and fine particles to pass from the stomach into the ducts but preventing the passage of material in the opposite direction. The muscular properties of the short extensions of the typhlosole into the openings of the ducts were noted by Graham (1949) who described them as performing 'slight twistings, expansions and contractions so that the whole structure has a gently lobed edge, the size and disposition of the lobes continuously undergoing slight changes'. *Zirphaea crispata* differs from *Cardium edule* and *Glossus humanus* in that the ciliary currents over the extension of the major typhlosole are directed away from the apertures of the ducts, but, as shown in fig. 10, c, the typhlosole is long and strap-shaped and an inhalant counterpart current almost certainly operates within the caecum as well as the non-ciliated portions of the main ducts. As suggested by Purchon (1955), the ciliary currents of *Zirphaea crispata* probably prevent blocking of the ducts and caeca, particles failing to remain in suspension in the fluid medium being returned to the stomach. Blocking is unlikely to occur in *Glossus humanus* where the stomach

undoubtedly possesses a more efficient sorting mechanism and only relatively fine particles are ingested (Owen, 1953).

Thus the pattern of ciliary activity within the eulamellibranch stomach is such that fluid and fine particles are conveyed to the proximity of the apertures of the caeca and ducts rather than directly into the ducts. Material is drawn into the non-ciliated portion of the main ducts by the inhalant counterpart current. Although the details are different, this is also the case in the Anisomyaria. The ciliary currents at the junction of a main duct with the stomach in *Ostrea edulis* and *Mytilus edulis* are represented diagrammatically in fig. 12 (in these species extensions of the major typhlosole do not project into the apertures of the ducts). The ciliated epithelium of the stomach

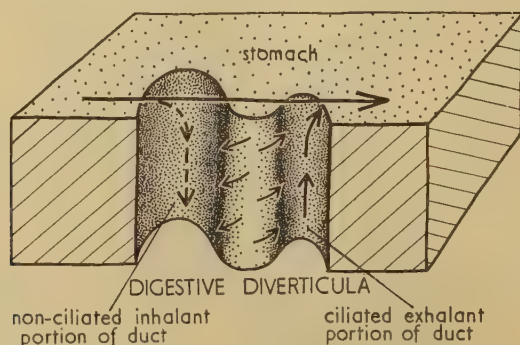


FIG. 12. Anisomyaria, junction of a main duct of the digestive diverticula with the stomach, shown diagrammatically. The unbroken arrows represent ciliary currents, the broken arrows an inhalant, counterpart current.

produces a current which sweeps across the mouth of the duct from the non-ciliated, inhalant side to the ciliated, exhalant side. Thus fresh fluid and particles are continuously drawn into the non-ciliated portion of the ducts while waste material is carried away from the ciliated portion.

In both the Anisomyaria and Eulamellibranchia yet another function may be attributed to the major typhlosole. At the junction of the mid-gut with the postero-ventral region of the stomach, the flap-like major typhlosole arches over the opening to the mid-gut, so preventing material from entering the latter except by the intestinal groove. It is interesting to note that in many species the opening of the oesophagus into the stomach is also protected, being slit-like and situated between fleshy, protuberant lips; a feature which reduces the possibility of regurgitation of the stomach contents. Thus, of the three possible exits from the stomach (the oesophagus, the mid-gut, and the ducts of the diverticula), two (the oesophagus and mid-gut) are 'guarded'.

A diagrammatic representation of the suggested functioning of the stomach and digestive diverticula of the Eulamellibranchia is shown in fig. 13. Particles entering the stomach from the oesophagus are subjected to a sorting mechanism and to the action of extracellular enzymes present in the stomach. Coarse particles are directed to the mid-gut by way of the intestinal groove and fine

particles and digested foodstuffs towards the openings of the caeca. Within the main ducts of the diverticula a continuous circulation is maintained by ciliary activity, material being carried out of the diverticula in the ciliated groove of the main ducts while free particles and digested foodstuffs are carried into the diverticula in the non-ciliated portion by the inhalant counterpart current. Fluid and fine particles are drawn into the tubules by way of the

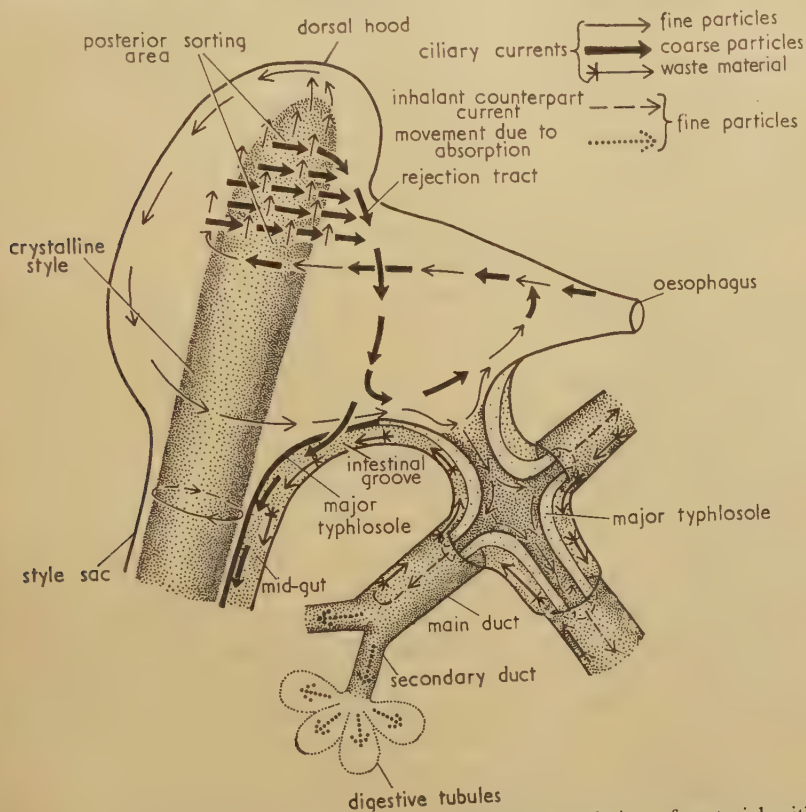


FIG. 13. A diagrammatic representation of the probable circulation of material within the stomach and digestive diverticula of most Eulamellibranchia. Heavy arrows represent coarse particles; fine arrows, fine particles; tailed arrows, waste material from the digestive diverticula; broken arrows, an inhalant counterpart current; dotted arrows, the absorptive activity of the cells of the tubules resulting in the movement of fluid and fine particles into the lumen of the tubules.

non-ciliated secondary ducts by the absorptive activity of the large vacuolated cells. Indigestible material and waste products are returned to the main ducts enclosed in large spheres formed by the fragmentation of the cells and are carried out of the diverticula by the cilia of the main ducts.

DISCUSSION

Many workers have attempted to discover the food of lamellibranchs by examining the stomach and faecal contents. Results have been confusing

owing largely to a failure to understand the functional morphology of the stomach of bivalves. Recently Mansour (1946a), after examining the stomach contents of a number of bivalves, claimed that animal plankton constitutes a much greater part of the food than is generally accepted and that this fact has been overlooked owing to the very rapid digestion of the animal food. As support for rapid digestion and in the belief that, as in many animals, food is propelled along the digestive tract of lamellibranchs at a rate which permits effective digestion, Mansour (1949) adds that the time taken for material to pass through the whole of the gut of *Unio prasidens* may be as short as 2 hours. Mansour-Bek (1948) tested filtered stomach juice for the presence of extracellular lipases and proteases. She obtained positive results but found that the strength of the enzymes as indicated by experiments did not correspond to the quick disintegration recorded by Mansour. Rosén (1949) also obtained only a weak protease reaction with filtered stomach juice. He suggests that this extracellular protease is much too weak to account for all the splitting of proteins and that the abundant occurrence of cathepsin bound intracellularly in the digestive diverticula indicates a considerable intracellular digestion of proteins.

The very rapid rate of digestion recorded by Mansour and the weak proteolytic and lipolytic enzymes present in the stomach would appear to be contradictory. The rate of passage of material through the gut of lamellibranchs, however, bears little relation to the effective duration of enzyme action (Yonge, 1937). The flap-like major typhlosole extending over the opening of the mid-gut prevents material entering the latter except by the intestinal groove, and this material is derived from the sorting areas of the stomach and the exhalant currents of the digestive diverticula (fig. 13). Material rejected by the sorting areas passes rapidly into the mid-gut and so to the anus and as a consequence of this may pass through the entire alimentary canal in a very short time (from personal observation material may pass through the entire gut of *Cardium edule* within 45 minutes). This feature of the lamellibranch stomach explains why several workers have observed that at least part of the 'food' of lamellibranchs passes through the alimentary canal unchanged. In addition to material which passes rapidly out of the stomach, particles too large to enter either the digestive diverticula or the intestinal groove may also be swallowed, since, under certain conditions, the gills and palps may pass into the mouth relatively large particles (Nelson, 1933). Yonge (1949) measured the maximum size of sand grains found in the pseudo-faeces, the stomach, and faecal pellets of *Tellina tenuis*. The measurements were 400μ , 320μ , and 80μ respectively, but particles of 80μ were rare in the faecal pellets, the majority not exceeding 40μ . Thus only particles a quarter of the diameter of the largest particles found in the stomach appear in the faeces. This retention of large particles in the stomach may explain the disintegrating zooplankton observed by Nelson (1933) and Mansour (1946a). The zooplankton organisms recorded by them were of relatively large size, e.g. nematodes, copepods, larvae of Crustacea, &c., and the flap-like major

typhlosole 'guarding' the entrance to the intestine would prevent such large objects entering the mid-gut (fig. 13). They must, therefore, remain in the gastric cavity until disintegrated. This could result from the triturating effect of the crystalline style (Yonge, 1949) aided possibly by any weak proteases and lipases present in the stomach cavity.

Mansour and Zaki (1946) injected into the blood of starved specimens of *Unio prasidens* a solution of chlorophyll, and subsequent examination revealed the presence of coloured globules in the large vacuolated cells of the tubules; similar globules were later found in the faeces. This was taken as evidence of the secretory function of the diverticula, the cells fragmenting and passing, together with their contained enzymes, into the stomach. Yonge (1946) remains convinced that the digestive diverticula are organs of intracellular digestion. He suggests that, while true extracellular lipases and proteases are absent from the gut of lamellibranchs, small amounts of these enzymes may occur owing to the presence in the lumen of numerous amoebocytes.

To test the phagocytic properties of the cells of the diverticula, Zaki (1951) fed *U. prasidens* with powdered gold-fibrin. No free gold particles were found in the cells of the diverticula and he assumed that these cells did not possess phagocytic properties. Similar techniques have been used by G. and S. Hörstadius (1940) using gold-gelatine and gold-fibrin, and Rosén (1941) using carbon-casein, the use of carmine and Indian ink having been criticized by Hirsch (1925) and Krijgsman (1928) on the grounds that they contain diffusible particles. Hörstadius's experiments with *Helix pomatia* were negative, free gold particles being absent from the cells of the digestive gland. This experiment was repeated by Rosén (1951) with similar results, but he found that '*the gold-fibrin had not passed up into the mid-gut gland*', probably because the gold-fibrin contained particles larger than the cells themselves. Feeding experiments with carbon-casein, whipped pigeon's blood, and edestin demonstrated that phagocytosis does take place in the cells of the digestive gland of *H. pomatia*. Rosén states that there is thus 'not only a lower critical limit but also an upper critical limit' for the size of particle ingested by phagocytosis. Certainly this is true of the *Anisomyaria* and *Eulamellibranchia*, where only very fine particles enter the tubules of the diverticula. Zaki (1951) in his account of feeding *Unio prasidens* with gold-fibrin does not give measurements of particle size. Furthermore, the animals were fed by mouth with a fine pipette, so increasing the possibility of using particles larger than those normally ingested. The positive results obtained with titanium dioxide provide clear evidence that the cells of the tubules ingest particles larger than 0.1μ .

Irrespective of whether the digestive diverticula are organs of secretion, feeding experiments with iron saccharate and other substances provide ample evidence that they are organs of absorption. Thus a two-way circulation is necessary to ensure the movement of the products of extracellular digestion into, and waste (with or without secretions) out of, the ducts of the diverticula. A continuous circulation is maintained within the main ducts solely as a result

of ciliary activity, but the entry of fluid into the blind-ending tubules is possibly a consequence of the absorptive functions of the large vacuolated cells. The entry of fresh fluid into the tubules will in this case be dependent on the rate of absorption, and though the resultant movement of fluid will be slow, solid particles able to remain in suspension in the fluid medium will also enter the lumen of the tubules. To prevent blocking of the tubules indigestible particles must be returned to the stomach on the way to the mid-gut and prevented from re-entering the tubules. As demonstrated by feeding experiments with titanium dioxide, the large vacuolated cells phagocytose solid particles present in the lumen of the tubules, the ingested particles being then concentrated in spherules and returned to the main ducts enclosed in large excretory spheres formed by the fragmentation of the cells. These spheres are conveyed out of the diverticula by the cilia of the main ducts. Thus in addition to an absorptive function, the large vacuolated cells of the tubules also serve an excretory function, which is an essential feature of their phagocytic properties.

The results obtained by Mansour-Bek (1948), Rosén (1949), and George (1952) indicate that weak extracellular proteases and lipases are present in the stomach although their source is uncertain. Preliminary extracellular digestion would certainly increase the amount of finely divided material received into the digestive diverticula for absorption and the completion of digestion intracellularly. The excretory spheres produced by the fragmentation of the tubule cells may contain proteases and lipases, and traces of these enzymes could be liberated into the stomach in this way, as suggested by Morton (1951) for the gastropod *Struthiolaria*. In both the Anisomyaria and Eulamellibranchia, however, such an explanation is improbable since the ciliary mechanisms of the stomach would appear to prevent material carried by the intestinal groove from returning to the general circulation within the stomach (Owen, 1953). Yonge's (1946) suggestion that traces of proteolytic and lipolytic enzymes are liberated into the lumen of the gut as a result of cytolysis of phagocytes would appear more likely.

This account of the functioning of the stomach and digestive diverticula of the Anisomyaria and Eulamellibranchia is intended to be of general interest, there being little doubt that individual species vary, particularly in the relative development and importance of muscular activity: e.g. the Anomalodesmata, in which the diverticula are relatively simple and sac-like. There would also appear to be some variation in the form and disposition of the major typhlosole, e.g. *Pandora* (Allen, 1954). There can be little doubt, however, that the organization of the stomach and diverticula in the majority of Anisomyaria and Eulamellibranchia is directed towards efficient intracellular digestion and that 'secretions' by the cells of the diverticula can play little part in the processes of digestion in these animals.

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Notes on the Mechanism of Food Movement in the Gut of the Larval Oyster, *Ostrea edulis*

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SUMMARY

The histology and mechanism of food movement in the stomach of the larval oyster are described. Cilia line the stomach, except the left wall, roof, and upper part of the right wall, which bear the smooth gastric shield. The diverticula contain non-ciliated absorptive cells and ciliated non-absorptive cells. A slender muscle passes round each diverticulum. Food is rotated in the stomach by the style, and also turned over in the sagittal plane. Particles are drawn into the diverticula and returned to the stomach by the rhythmic expansion and contraction of the diverticula.

INTRODUCTION

MUCH is now known of the structure, histology, and functioning of the gut in the adult oyster and other lamellibranchs. In the larval oyster, also, the general structure and function have been described (Horst, 1883; Yonge, 1926; Erdmann, 1935), but the existing accounts need some additions and corrections, especially with regard to the manipulation of the food once it has reached the stomach. The present paper deals with the larva of *Ostrea edulis* L., which was studied during breeding experiments at the Marine Station, Millport.

METHODS

In order to follow the movements of particles within the gut, larvae were kept in sea-water to which was added titanium dioxide (Acheson Colloids Ltd.), or cells of the flagellate *Isochrysis galbana* Parke. Larvae were also fixed in Bouin, in the expanded state, by first using propylene phenoxetyl (Owen, 1955) as a narcotizing agent. Paraffin sections were cut at 5μ and 10μ and stained with Heidenhain's haematoxylin, followed by acid fuchsin or orange G as counterstain.

STRUCTURE OF THE GUT

The gut has already been described and illustrated (see figures in Yonge, 1926, and Erdmann, 1935). The long oesophagus leads into the anterior end of the stomach, and from the posterior end of the stomach the style sac projects backwards. From the posterior end of the right side of the stomach the mid-gut passes round the end of the style sac and turns forward to make an anterior loop lying to the left of the stomach. The rectum runs almost straight back to the anus. The diverticula lie one on each side of the stomach and open separately into its floor.

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The oesophagus is uniformly ciliated and of simple structure, but the stomach is more complex. At its anterior end immediately above the opening of the oesophagus is an area with long cilia, and the lower part of the right wall also bears cilia. On the upper part of the right wall, on the roof, and on the whole of the left wall, however, the epithelium of the stomach is modified and instead of having cilia is covered by the smooth gastric shield (fig. 1). The existence of this structure in the larva has already been noted by Erdmann

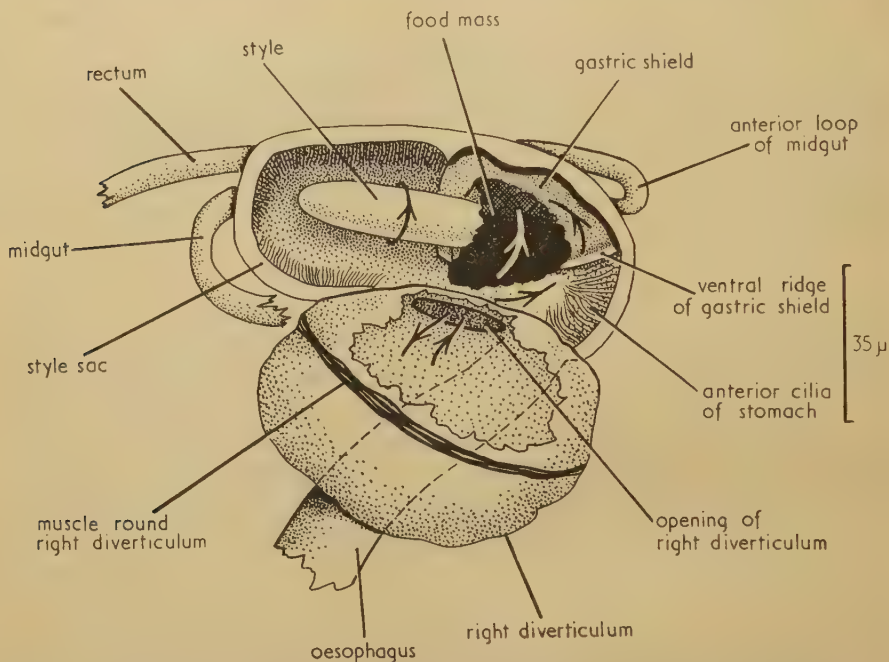


FIG. 1. Gut of larval oyster seen from the right; partly reconstructed from sections. The right wall of the stomach and part of the right wall of the right diverticulum have been cut away. Arrows show the direction of movement of the food and the rotation of the style.

(1935), but he stated that the shield was on the right and dorsal walls of the stomach. He deduced its position from a reconstruction of sections (see his Abb. 5, p. 20), but the true situation of the gastric shield on the left and dorsal walls can be determined by using as landmarks the opening of the midgut on the right, and also the anterior loop of the midgut which lies to the left of the stomach. In Erdmann's own figure (Abb. 18, Taf. 3) the shield is seen to be on the same side as the anterior loop of the midgut, i.e. on the left. The gastric shield is formed of the specially thickened cuticular border of the epithelial cells. Part at least of the left ventral border of the shield is further thickened and inrolled as a distinct ridge (see fig. 1). In a transverse section this ridge appears as a hook-like structure projecting into the lumen of the stomach. The cells underlying the gastric shield are in other respects similar to those of the rest of the stomach and of the style sac. The style sac is uniformly ciliated but

the cilia of the dorsal surface are stouter than those of the ventral surface and there appears to be a small apical area of the sac which lacks cilia. The style, lying within the sac, projects forward into the stomach. A small central strip on the floor of the stomach is ciliated and on each side of this strip lie the single openings to the diverticula. Each diverticulum in the larva is simple and sac-like, unlike the much divided structure of the adult. The cells of its walls are of two kinds, absorptive and ciliated (fig. 2). Most of the cells are absorptive, and these are cubical with lightly staining cytoplasm containing a few vacuoles. Material within these vacuoles is presumed to be absorbed food, as Yonge (1926) has shown that absorption occurs exclusively in the diverticula. The nuclei of these cells, often deformed by pressure of the food vacuoles,

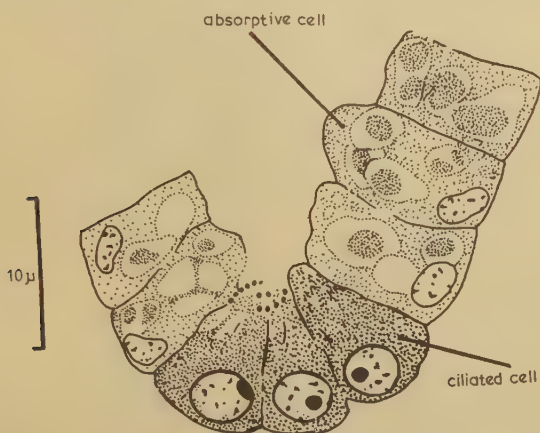


FIG. 2. Part of the wall of a diverticulum, in section.

contain only scattered chromatin and no large nucleolus. The remaining cells occur in small groups called by Yonge (1926) crypts of young cells. These cells have more deeply staining cytoplasm than the absorptive cells. They are also distinguished by their nuclei which have a large nucleolus and are spherical, there being no vacuoles in the cytoplasm to cause nuclear deformation. Although cilia have not been seen with certainty on these cells, their apical ends bear rows of granules deeply staining with iron haematoxylin and having the appearance of basal granules. It is therefore highly probable that they are the ciliated cells of the diverticula, especially as cilia have been demonstrated by Owen (1955) on similar cells in the tubules of the adult diverticula. In the larva, however, cilia are not responsible for the movements of food particles into and out of the diverticula, these movements resulting from muscular activity. Fig. 3 represents an obliquely transverse section through a larva, and shows a slender strand of muscle fibres passing round the outside of each diverticulum in an antero-ventral to postero-dorsal direction. The strand of muscle seems to come into contact with the posterior retractor muscle of the velum, but its relationship to that muscle is obscure. There may possibly be

other fine strands of muscle round the diverticula, but no others were seen. Both the midgut and the rectum are ciliated, and at the entrance of the midgut to the stomach the cilia are especially large.

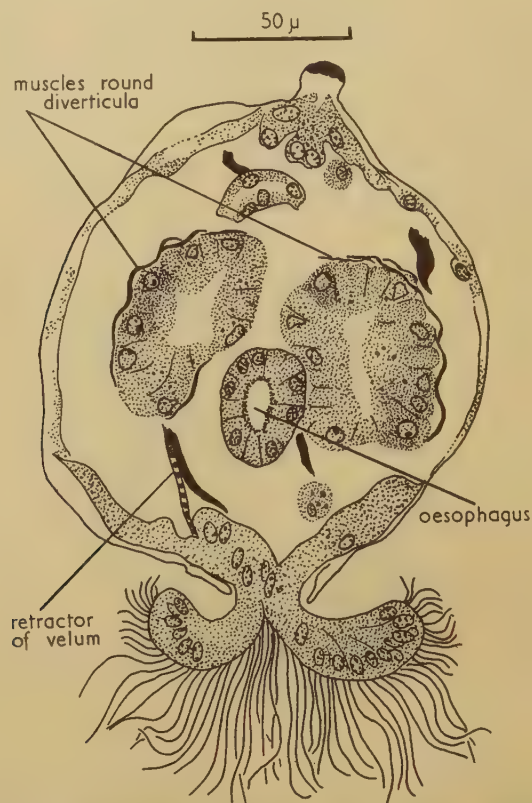


FIG. 3. Obliquely transverse section through a larva, to show the muscles round the diverticula.

MECHANISM OF FOOD MOVEMENT

Food particles collected by the cilia of the velum and mouth are passed by the oesophageal cilia into the front of the stomach. Here they accumulate round the end of the style as a conical mass which is rotated by the action of the style revolving under the influence of the cilia of the style sac. The cilia of the right wall of the stomach probably also help to rotate the food. In all oyster larvae which I have examined the food mass revolved in a clockwise direction as viewed from the anterior end; that is, the movement of particles is up across the right wall and down across the left wall. It was often noticed that rotation of the food mass was slowed down or stopped, as if by an obstruction, in the lower left part of the stomach. This obstruction is, in fact, the ventral ridge of the gastric shield. Yonge (1926) states that in some larvae which he observed the rotation was clockwise and in others anti-clockwise,

although constant in any one larva. As mentioned above, I have seen only clockwise rotation, and anti-clockwise rotation might be rendered difficult by the shape of the ventral ridge of the gastric shield, but clockwise rotation is apparently adapted to that shape. The gastric shield is perhaps useful in breaking up food organisms as they are pressed against it in rotating, although such a process hardly seems necessary with the small delicate flagellates which are known to constitute the food of the larvae (Cole, 1937; Bruce, Knight, and Parke, 1940). The shield may also serve, as in the adult, to grind the end of the style.

In addition to rotating about the axis of the style, the food mass is also constantly turned over by a sort of rotation in the sagittal plane. This is accomplished by the cilia of the ventral strip of the stomach driving particles forward and by the long cilia of the anterior end of the stomach passing them up to the dorsal side of the food mass (fig. 1).

Food particles pass from the stomach into the diverticula, not by ciliary activity, but by the rhythmic expansion and contraction of the diverticula themselves, brought about by the action of the slender muscles which pass round them. This pulsation was seen only when the larva was in the swimming position with the velum protruded and never when the body was retracted between the shells. In different larvae the rate of pulsation varied between 5 and 22 contractions per minute, and was usually between 18 and 21. When the diverticula dilate food particles are drawn in, and when they contract they are expelled to rejoin the rotating food mass in the stomach. Larvae seen from the dorsal side showed that the diverticula do not pulsate in unison, but alternately. This may be advantageous by preventing undue pressure-changes within the stomach which might result from the simultaneous action of the two diverticula. It may also help each diverticulum to expand if its muscular relaxation coincides with the increased pressure due to the contraction of the other diverticulum. But it is difficult to decide to what extent pressure-changes of this kind would be transmitted from one to the other.

Particles are drawn off from the posterior end of the stomach by the cilia at the mouth of the midgut. As there appears to be no sorting mechanism in the larval gut, other than the exclusion of large particles by the small diameter of the mouth and oesophagus, it is a matter of chance whether material drawn off into the midgut and thence passed to the rectum has been in the stomach for a short or a long time, and therefore to what extent it has been subjected to digestion.

DISCUSSION

The rotation of food round the end of the style and its turning over in the sagittal plane ensure thorough mixing and uniform treatment of all material taken in. The direction of rotation which I have observed is the same as that recorded in adult lamellibranchs (Nelson, 1918; Yonge, 1926, 1949, 1951a, 1951b; Owen, 1953; Allen, 1954), and the position of the gastric shield is

similar. There is therefore continuity of structure and function from the larva to the adult.

The larval diverticula also have a certain resemblance to those of the adult, although much simpler in form. Similar absorptive and ciliated cells occur, and the function of the diverticula is evidently comparable in both stages of the oyster. But there may be important differences in the mechanism by which particles are exchanged between the stomach and the diverticula. Owen (1956) observed no contraction of the muscles which envelop the tubules of the diverticula of the adult oyster, although he suggests that the system of longitudinal fibres may 'from time to time, contract slowly to empty the contents of the tubules . . .'. He concludes that solid particles and liquid are passed into the tubules by the ciliary activity of the ducts, in which there is also a compensating flow in the opposite direction. In the larva there are no ducts to the diverticula, which open directly into the stomach, and the cilia of the diverticula probably serve only to agitate particles already present in the lumen. The pumping action of the diverticula produced by their own muscles is a much more efficient way of emptying and refilling these sac-like structures than a ciliary mechanism would be. It is uncertain how far the simple and active muscles of the larval diverticula are comparable with the complex and apparently inactive system of the adult.

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The Posterior Lobes of the Brain of *Nephtys* and the Mucus-Glands of the Prostomium

By R. B. CLARK

(From the Department of Zoology, University of California, Berkeley, California)

With one plate (fig. 4)

SUMMARY

A pair of lobes is attached to the posterior margin of the supra-oesophageal ganglion of some species of *Nephtys*. They are filled with large, vacuolated mucus-cells similar in appearance and histochemical properties to those found in the anterior part of the prostomium, in the lateral walls of it, and in the parapodia. The mucus-cells of the posterior lobes, and sometimes those of the anterior prostomial group also, have long necks which run in a tract to the epidermis, where they open to the exterior. When this is so, they replace the epidermal mucus-cells found in the lateral walls of the prostomium in species lacking posterior lobes. It is suggested that there has been a centripetal migration of epidermal mucus-cells into the posterior lobes and to the anterior prostomial group, a phenomenon closely paralleling that found in the evolution of the nermertean cerebral organ. In one species, *Nephtys cirrosa*, the posterior lobe cells appear to have undergone a further modification, for they are much more closely integrated with the nervous system and differ in appearance from those of other species, coming to look, at least superficially, like the majority of neurosecretory cells in the brain.

MOST families of polychaetes are homogeneous, but there is none more so than the Nephtyidae. The smallest species is only a few millimetres long, the largest a foot long, but apart from this size-difference, one member of the family looks much like another. Most systematists (e.g. McIntosh, 1908; Fauvel, 1923) have regarded the family as monogeneric, though in the most recent monograph (Hartman, 1950) it has been divided into three genera, one of them comprising a single species. Whether the family should properly be regarded as mono- or trigeneric, the differences separating genera and species are trivial. The only characters on which taxonomists can rely are the number and disposition of the branchiae, the form of the parapodial lobes and chaetae, and the number of papillae on the proboscis. This uniformity of external morphology is undoubtedly a reflection of the fact that all the species live in much the same habitat and, from what little is known of them, have similar habits. *Nephtys* lives in intertidal or sublittoral sand or mud; it does not occur among rocks or debris. It probably does not form a permanent burrow, but crawls around in the substratum which it may leave for spawning and other excursions since it is an active swimmer.

The structure which *a priori* one would expect to vary least in such a family is the nervous system. Not only is this regarded as a comparatively conservative part of the animal, so far as evolution is concerned; but, if the behaviour is more or less the same from one species to another, one would expect that even

in detailed structure the brains of all the nephtyids would be the same. This is not the case, however. Even in gross morphology the supra-oesophageal ganglion and its associated structures are extremely variable and there are many obvious differences in the minute structure of the ganglion in different species. In this paper attention will be directed towards a pair of

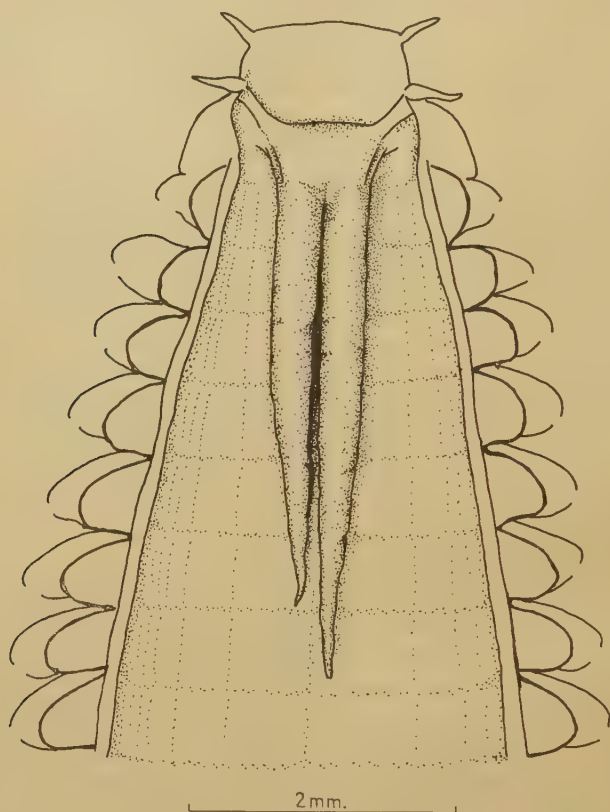


FIG. 1. Supra-oesophageal ganglion and posterior lobes of *N. californiensis*. Dissection from the ventral side.

posterior lobes of the supra-oesophageal ganglion which are present in some species but not in others.

There has been a handful of papers describing the central nervous system of *Nephtys* (Delle Chaije, 1825; Quatrefages, 1850; Claparède, 1868; Ehlers, 1864-8; Pruvot, 1885; Schack, 1886; de Saint-Joseph, 1894), and these are full of contradictions and discrepancies. The contradictions spring from the fact that the authors expected that the nervous system would be identical in all nephtyids and engaged in controversy with others who had examined different species of *Nephtys* with different results. The situation has not been improved by an apparent confusion or misidentification of species (Rullier, 1947; Clark, 1956c). Ehlers (1864-8), and Schack (1886) described a pair of cylindrical lobes extending from the posterior margin of the supra-oesophageal ganglion

of *N. caeca* to the fifth segment. Quatrefages (1850) and de Saint-Joseph (1894), who also examined this species, denied their existence. There was general agreement that there were no such lobes in *N. hombergi* and since it was assumed that the brain of a polychaete would not vary so greatly within a single genus, Ehlers and Schack were presumed to have been mistaken in their observations. In fact, they were right and Quatrefages and de Saint-Joseph were wrong. Apart from the (incorrect) observation by Schack that the posterior lobes are filled with exceptionally large ganglion cells, the lobes have not been investigated histologically and the purpose of the present paper is to discuss the nature and homologies of the lobes and their distribution in the Nephtyidae, and to suggest how they may have evolved. This has demanded a comparative study of all the available species of *Nephtys*. There are some 70 species in the family and it has not been possible to collect more than a small number of them. I have been able to supplement these collections with specimens from museums, although these are not ideally fixed for histological work and the examination of them has been comparatively superficial.

MATERIALS AND METHODS

This study has been based on an examination of the species of *Nephtys* and *Aglaophamus* listed below. The two genera are regarded as sub-genera of *Nephtys* by some systematists. I have had no opportunity to examine *Micro-nephtys*, the third genus in the family. The species marked with an asterisk have been taken from museum collections and are therefore inappropriately fixed for careful histological work; the rest have been collected and fixed in Bouin's fluid made up in sea-water, as a general rule. *N. californiensis* is readily available on the central California coast and has accordingly been used for experimental purposes. It has been fixed in a variety of ways: Bouin's fluid, picroformol, Heidenhain's 'Susa', Zenker-formaldehyde, Zenker-acetic, formalin, and absolute methyl alcohol. All but the last two fixatives have been made up in sea-water mixed with distilled water so as to make the fixative approximately isotonic with sea-water, as a means of reducing shrinkage.

Species examined:

N. caeca (Fabricius)
N. caecoides Hartman
N. californiensis Hartman
N. cirrosa Ehlers
 **N. cornuta* Berkeley and Berkeley
N. cornuta franciscana Clark and Jones
N. ferruginea Hartman
 **N. glabra* Hartman
N. hombergi Aud. and Edw.
N. incisa Malmgren

N. longosetosa Oersted
 **N. magellanica* Hartman
N. parva Clark and Jones
N. picta Ehlers
N. punctata Hartman
 **N. rickettsi* Hartman
 **N. squamosa* Hartman
 **Aglaophamus dicirris* Hartman
 **A. erectans* Hartman
 **A. virginis* (Kinberg)

N. rickettsi is synonymous with *N. discors* Ehlers according to Pettibone (1954).

The gross morphology of the anterior nervous system with its associated structures has been determined by dissection. As the entire dorsal part of the supra-oesophageal ganglion is usually in contact with the cuticle of the pro-stomium, it is most convenient to make lateral incisions of the body-wall as far forward as the first segment, to reflect the dorsal body-wall forwards and to dissect the brain from its ventral surface.

Transverse and frontal serial paraffin sections have been cut at 7 or 10 μ and stained as a matter of routine with paraldehyde fuchsin. This technique has been developed by Gomori (1950), Halmi (1952), and Dawson (1953) for the study of neurosecretory products. I have used Gabe's (1953) method of preparing the paraldehyde fuchsin. The final method is thus:

1. Remove paraffin and hydrate sections.
2. Refix in Zenker-formaldehyde for 1–2 hours.
3. Lugol's solution, 5 minutes.
4. Rinse in water.
5. 5% sodium thiosulphate, 2 minutes.
6. Rinse in water.
7. Oxidize in acid permanganate 1 minute.

Potassium permanganate	.	.	3 g
Conc. sulphuric acid	.	.	3 ml
Distilled water	.	..	1,000 ml
8. Rinse in water.
9. Decolorize in 2.5% sodium bisulphite.
10. Wash in water.
11. Stain in paraldehyde fuchsin, 10 minutes.

The paraldehyde fuchsin is made according to the directions given by Gabe (1953). The stock solution consists of a 0.75% solution of paraldehyde fuchsin in 70% alcohol. The staining solution found best for polychaete material is:

Stock solution	.	.	15 ml
70% alcohol	.	.	150 ml
Glacial acetic acid	.	.	2 ml

(For vertebrate material the stock solution should be diluted only 3 or 4 times with 70% alcohol.)

12. Wash in two changes of 95% alcohol.
13. Rinse in 0.25% hydrochloric acid in absolute alcohol, 15 seconds.
14. Rinse in distilled water.
15. Mordant in phosphotungstic-phosphomolybdic acid for 10 minutes.

Phosphotungstic acid	.	.	4 g
Phosphomolybdic acid	.	.	1 g
Distilled water	.	.	100 ml
16. Rinse in water.
17. Counterstain 1 hour.

Light green (fast green)	.	.	0.4 g
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Orange G	1.0 g
Chromotrope 2R	0.5 g
Glacial acetic acid	1.0 ml
Distilled water	100 ml

18. Rinse in 2% acetic acid in 95% alcohol.

19. Dehydrate rapidly, clear, and mount.

Best results have been obtained with material fixed in Bouin's fluid, but mercuric fixatives are also suitable, though they usually require a stronger solution of paraldehyde fuchsin (dilute the stock solution only 4–5 times with 70% alcohol, instead of 10 times). The times suggested above are for Bouin-fixed material. Steps 2–5 may be omitted, but mordanting with Zenker-formaldehyde improves counterstaining. Although lengthy, this technique has the advantage of being a quite delicate stain for nervous tissue and also of giving excellent colour differentiation of various tissues.

Other staining techniques have been used, including Mallory triple stain and a series of histochemical techniques, which will be referred to in the text where appropriate.

ANATOMY AND HISTOLOGY OF THE POSTERIOR LOBES OF *NEPHTYS CALIFORNIENSIS*

The supra-oesophageal ganglion of *N. californiensis* is a trapezoidal structure lying in the posterior part of the prostomium and the anterior part of the first segment (fig. 1). It is in contact with the prostomial cuticle and in segment I is suspended beneath the epidermis. It is bounded by a connective tissue sheath which is continuous with the basement membrane of the epidermis (fig. 2). Attached to the posterior margin of the ganglion, continuous with it and enclosed within the same membrane, there are two tapering cylindrical processes, the posterior lobes. They extend caudally as far as segment VII or VIII. Occasionally they extend only into segment VI and sometimes they reach segment IX. In living or freshly killed worms the posterior lobes are translucent and whitish; the supra-oesophageal ganglion is dark. In preserved material the ganglion becomes opaque, dull white. In all events, the lobes can be clearly distinguished from the ganglion proper even in a cursory examination.

The posterior lobes are filled with large, irregularly shaped and highly vacuolated cells (fig. 3). These cells vary in size, but are usually about 100μ long and 40μ wide. The vacuoles may be small and numerous, as in fig. 3, or they may apparently coalesce to form one or two large vacuoles which occupy most of the cell. When this is so, the nucleus is frequently to one side of the cell and thin strands of cytoplasm cross the cell between the vacuoles. Alternatively there may be a single vacuole, opening into the neck of the cell. The vacuole contains granules, and the cell therefore has the same appearance as the prostomial mucus-cells (fig. 4, A). Because of their high degree of vacuolation, these cells in the posterior lobes are difficult to fix satisfactorily. After fixation by mercuric chloride the material in the vacuoles has a reticulate

appearance, but after Bouin's fixation the vacuoles can be seen to be filled with numerous granules or globules of secreted material (fig. 4, B).

The nuclei of posterior lobe cells are roughly oval, approximately $10 \times 15 \mu$, and are frequently irregular in outline. Often the nuclear surface has a number

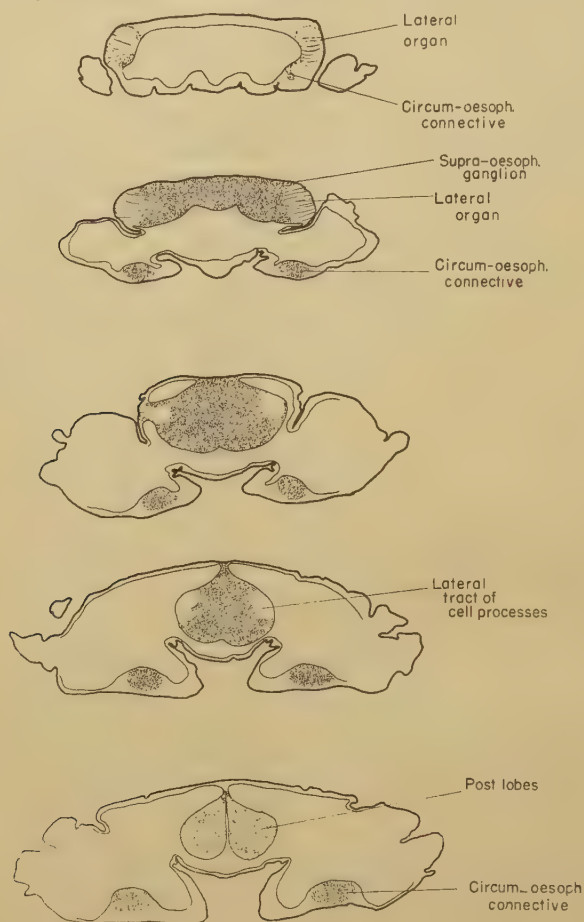


FIG. 2. Series of transverse sections through the prostomium and first segment of *N. californiensis*, to show the relation between the nervous system, the posterior lobes, and the epidermal basement membrane.

of projections and bumps on it. This does not appear to be a fixation artifact because the nuclei have the same appearance whatever fixative is used. It is sometimes found that nuclei of cells undergoing great activity are irregular in outline, as for example in some neurosecretory cells (Scharrer and Scharrer, 1954). There is usually a single large nucleolus which is very conspicuous, but in a minority of the cells (possibly 5% of them) there are two nucleoli, although in other respects these cells resemble the others in the posterior lobes.

The posterior lobe cells are drawn out and have long necks. These cell

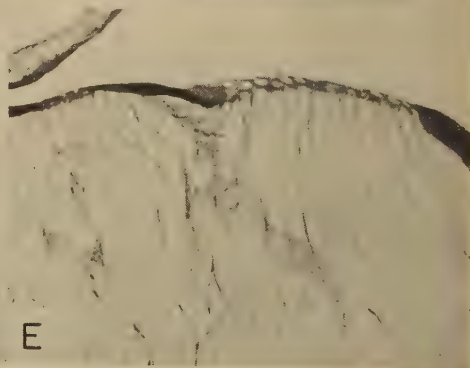
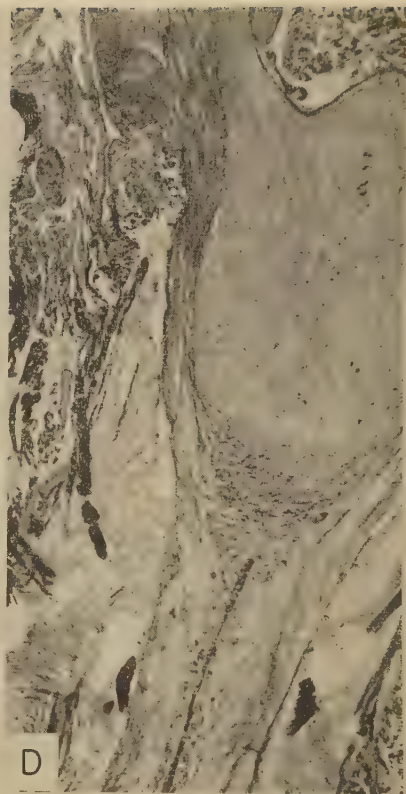
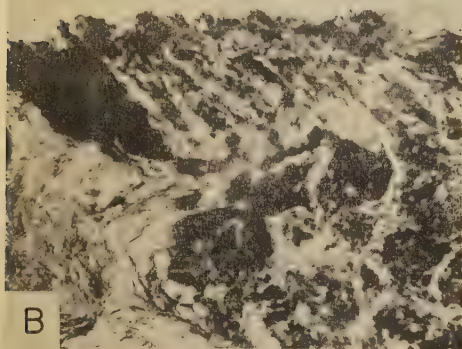
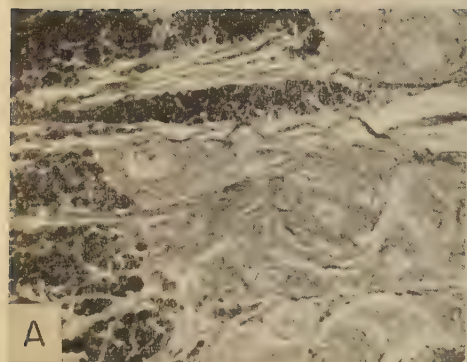


FIG. 4
R. B. CLARK

processes collect together and run in a tract along each side of the supra-oesophageal ganglion (figs. 2 and 4, D) to the anterior part of it where the circum-oesophageal connectives originate. There they turn sharply through a right angle and run perpendicularly to the lateral walls of the prostomium (fig. 5). The cell processes running to the prostomial walls are very distinctive



FIG. 3. Cells from the posterior lobes of *N. californiensis*. Camera lucida drawing.

and will be called the lateral organs for the sake of convenience, though they cannot properly be regarded as constituting an organ any more than, for instance, can the sinus gland of the crustacean eye-stalk. The ends of the

FIG. 4 (plate). A, mucus-cells of the anterior prostomial group of *N. caeca*. The long necks of the cells are filled with fuchsinophil granules. Bouin; paraldehyde fuchsin; 7μ paraffin sections. No filter.

B, posterior lobe cells of *N. longosetosa* filled with strongly fuchsinophil granules. Granules can be seen filling the cell processes which run towards the upper left-hand corner to the lateral tract. Bouin; paraldehyde fuchsin; 7μ paraffin sections. Wratten 58 filter.

C, posterior lobe cells of *N. cirrosa* showing the large non-secretory matrix cells, neuroglial fibres, and, along the upper edge, small secretory cells filled with fuchsinophil material. Bouin; paraldehyde fuchsin; 7μ paraffin sections. Wratten 58 filter.

D, frontal section through the prostomium and anterior segments of *N. californiensis*. The supra-oesophageal ganglion occupies the upper right-hand half of the photograph; cell processes from the posterior lobes run along the sides of the ganglion to the lateral organ in the upper left centre of the figure. Zenker-formol; paraldehyde fuchsin; 7μ frontal sections. Wratten 58 filter.

E, lateral organs of *N. ferruginea*. The cell processes run to the dark-staining cuticle which can be seen to be perforated over the ends of them. Bouin; Mallory triple stain; 10μ paraffin sections. Wratten 25 filter.

processes penetrate into and possibly through the cuticle overlying them. The prostomial cuticle is fairly thick (16μ) in most places, but over the lateral organs it is reduced to a thickness of 2.5μ and over the terminations of the cell processes it is either absent altogether, or else is so thin that it cannot be detected by ordinary histological methods. When the cuticle is stripped from

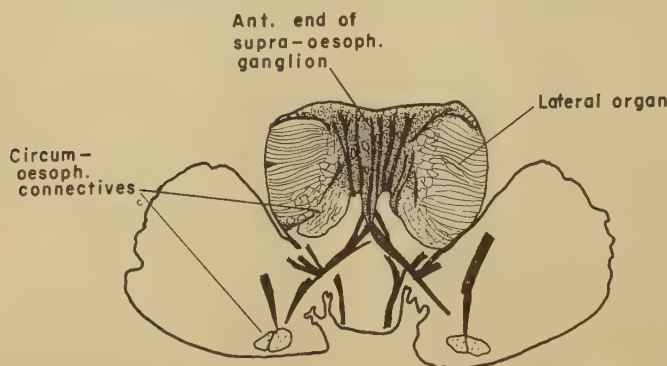


FIG. 5. Transverse section through the prostomium of *N. ferruginea*, showing the lateral organs at their greatest development.

the sides of the prostomium it is seen to be peppered with perforations marking the ends of the cell processes of the lateral organs (fig. 4, E). The lateral organs extend along the sides of the prostomium in front of the supra-oeso-

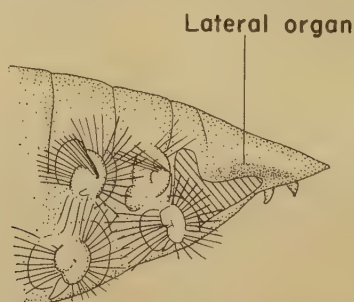


FIG. 6. Lateral view of the prostomium and anterior segments of *N. californiensis*. Cross-hatched area indicates the attachment of the first parapodium which has been removed to expose the area occupied by the lateral organ.

phageal ganglion, almost to the anterior antennae at the antero-lateral corners of the prostomium (fig. 6).

The lateral organs are not, in this species, derived entirely from the posterior lobe cells. A second group of vacuolated cells, of exactly the same form and with the same staining properties as those in the posterior lobes, are situated in the anterior part of the prostomium (figs. 7 and 4, A). A fine bundle of cell processes extends from these to the anterior part of the lateral organ, though the bundles of processes are not as distinct as those running along the sides of the supra-oesophageal ganglion from the posterior lobe cells. Further,

there are vacuolated cells located along the tract of processes, so that nowhere is there a distinct demarcation between the cell body region and the tract of processes as in the posterior lobes. There are thus anterior and posterior lateral organs, continuous with each other, but derived from prostomial and posterior lobe cells respectively.

The entire system of vacuolated secretory cells and their processes is epidermal, as is the nervous system. They are all bounded internally by the

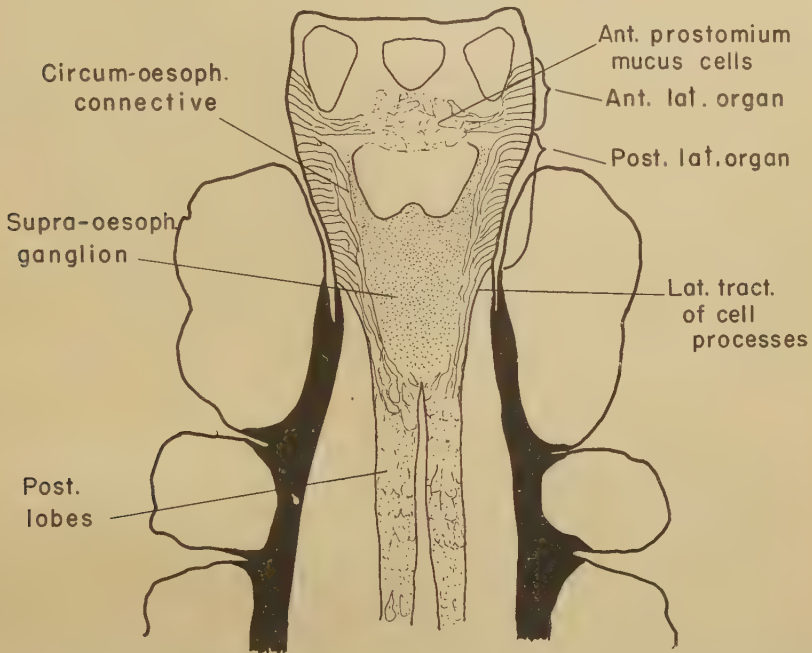


FIG. 7. Composite frontal section through the anterior end of *N. californiensis* to show the relation between the posterior lobes, the lateral tracts, the lateral organs, and the prostomial mucus-cells. Based on camera lucida drawings.

basement membrane of the epidermis or by extensions of it. The posterior lobes and the lateral tracts formed by the processes of the cells filling them are enclosed within the same connective tissue membrane as the supra-oesophageal ganglion and have the same thin, cellular, pericapsular sheath on its outer surface (Clark, 1956a). The vacuolated cells are sharply marked off from the nervous tissue of the ganglion by a dense mass of neuroglial fibres, and a similar but thinner layer of neuroglial fibres separates the lateral tracts from the ganglion, although they are all within the same connective tissue sheath (fig. 4, D). Neuroglial fibres penetrate between the cell processes in the lateral tracts and occasional neuroglial cell bodies can be seen scattered among the cell processes in the lateral tracts and lateral organs. Neuroglial fibres also penetrate into the posterior lobes, though they are not numerous, and a few of the cell processes from the posterior lobes run through the neuroglial mass

at the posterior end of the ganglion. The neuroglia separating the posterior lobes and lateral tracts from the ganglionic material appears to be of a different constitution from the neuroglia in the ganglion, though no morphological differences can be seen. Under some (undetermined) conditions of fixation or staining, the neuroglia at the posterior end of the ganglion stains with the orange G while the neuroglia of the ganglion proper takes up light green in the counter-stain, but usually both stain in the same way.

From anatomical considerations alone it would be reasonable to suppose that secretions produced in the vacuolated cells of the posterior lobes and the anterior prostomial cells reach the exterior by way of the lateral organs. This is indeed so, for if the worms are roughly handled in the process of fixation, the cell contents are frequently extruded. Some worms were narcotized and most of the body-wall was trimmed away in the hope of improving fixation of the posterior lobes. The posterior lobes and the lateral organs were found to be empty and only a small quantity of fuchsinophil material was found on the cuticle over the lateral organs. Other worms which have been less severely handled before fixation have shown empty posterior lobe cells, but with the secretion concentrated in the lateral organs and penetrating through the cuticle on to its outer surface.

THE NATURE OF THE SECRETION

The secretory cells of the posterior lobes, the lateral organs, and the anterior prostomial group of cells are all PAS-positive. They therefore fall into the group of tissues containing polysaccharides, mucopolysaccharides, mucoproteins, and glycolipids (Pearse, 1953). The presence of glycogen can be ruled out by preliminary digestion with diastase, which does not change the PAS reaction. The cells also give a positive reaction with paraldehyde fuchsin, mucicarmine (Southgate, 1927), dialysed iron (Hale, 1946), and alcian blue 8GS (Steedman, 1950), and show metachromasia with toluidine blue. These techniques were carried out on paraffin sections of *N. californiensis* fixed with formalin and also on sections fixed with absolute methyl alcohol. The results were identical with both fixatives.

These histochemical tests, while not conclusive, suggest that the posterior lobe cells produce a mucoid material of some sort which is probably an acid mucopolysaccharide. The epidermal mucus-glands of the parapodia have an appearance similar to those of the posterior lobes and the anterior prostomial group of cells, and have the same staining reactions when these tests are applied to them. Thus although the posterior lobe mucus cells are anatomically specialized, they do not appear to produce a secretion different from that of the simple epidermal mucus-cells of other parts of the body. This is not the general experience, for specialized mucus-cells in various parts of the body of an animal frequently produce different sorts of mucus (see, for example, Gomori, 1954) and the differences are commonly sufficiently great to be detected by the methods employed here.

THE COMPARATIVE ANATOMY OF THE POSTERIOR LOBES AND THE PROSTOMIAL MUCUS-GLANDS OF THE NEPHTYIDAE

Posterior lobes, similar morphologically and histologically to those described in detail in *N. californiensis*, also occur in *N. caeca*, *N. caecoides*, *N. ferruginea*, *N. longosetosa*, *N. parva*, and *N. punctata*. The degree of development of the lobes varies considerably from one species to another. In *N. parva*, a small worm, the lobes are so narrow that only 3 or 4 cells can be seen in cross-section, but the lobes extend to segment XI. In *N. longosetosa* they extend to segment VII, in *N. caeca* only to segment V. The longest lobes I have seen are those of *N. caecoides*, in which they extend from the supra-oesophageal ganglion in segment I to segment XV. In all these species the lobes are filled with vacuolated cells similar to those of *N. californiensis*, and the presence of lobes of this sort involves also the presence of lateral tracts of cell processes running to the lateral organs.

In a second group of worms, viz. *N. cornuta*, *N. cornuta franciscana*, *N. hombergi*, *N. incisa*, *N. picta*, *Aglaophamus dicirris*, *A. erectans*, and *A. virginis*, there are no posterior lobes filled with vacuolated mucus-cells. The posterior end of the supra-oesophageal ganglion may be bifurcate, however, and give the superficial appearance of lobes. This is undoubtedly the basis of Rullier's (1947) statement that 'ces lobes postérieurs existent chez toutes les *Nephtys* que j'ai étudiées. Ils sont très longs chez *N. caeca*, moins développés chez *N. cirrosa* et très courts chez *N. hombergi* et *N. hystricis*. Il y'a donc tous les termes de passage.' I have not examined *N. hystricis*, but the supra-oesophageal ganglion of *N. hombergi* and that of all the other species in this group, whether bifurcated posteriorly or not, ends in a mass of neuroglial fibres similar to those separating nervous tissue from glandular tissue in *N. californiensis* and the species of the first group. *N. cirrosa* represents a special case which will have to be discussed in greater detail below. It has lobes unlike those of any other species I have seen.

The mucus-glands of the prostomium are as variable in their arrangement and disposition as the posterior lobes. The locations of these glands in the Nephtyidae are in a group in the anterior median part of the prostomium, along the lateral walls of the prostomium, and in the posterior lobes. The mucus-cells in the lateral walls of the prostomium can be divided into two groups, anterior and posterior, on the basis of their fate in certain species. The division between the anterior and posterior groups of lateral mucus-cells can be set at the level of the posterior antennae, though no anatomical division between the two groups can be made in those species in which both are present, because they grade into each other. The two groups correspond to the anterior and posterior divisions of the lateral organs of *N. californiensis*.

The anterior median prostomial group of mucus-cells is present in all species. These cells may have long necks and open to the lateral walls of the prostomium by way of the anterior lateral organs, as in *N. californiensis*, *N. caecoides*, *N. longosetosa*, and *N. cornuta*, or they may open directly to the

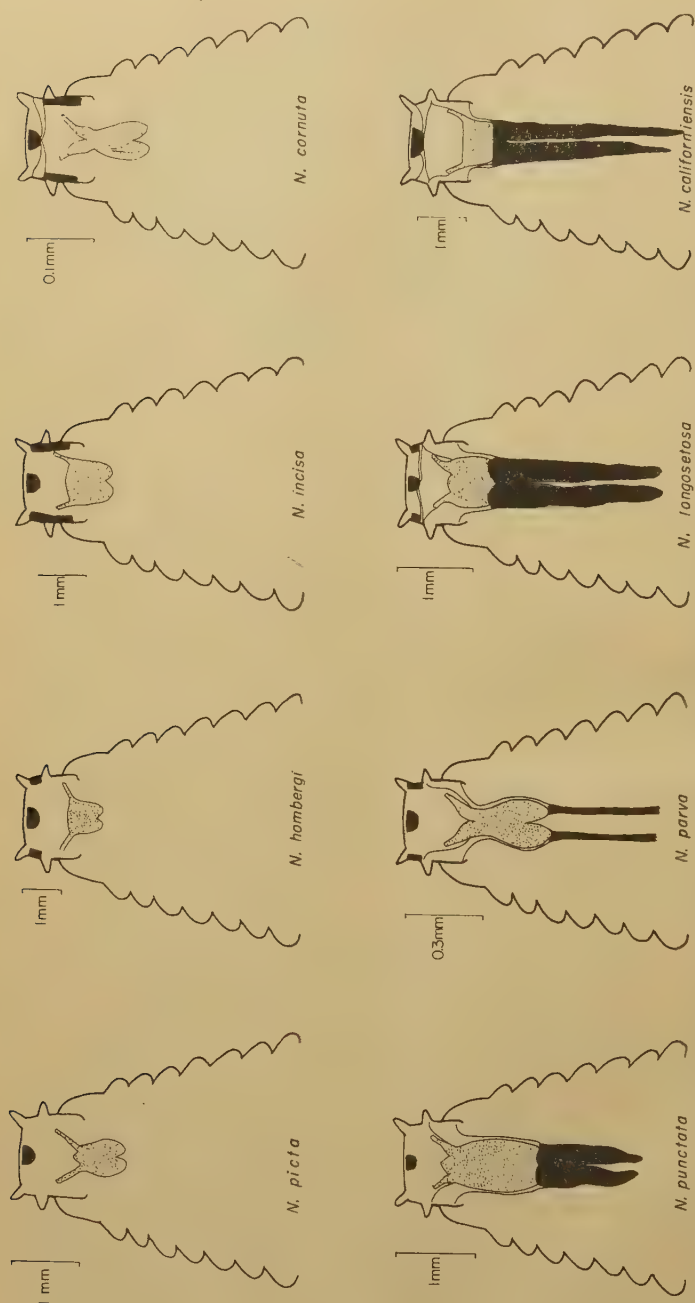


FIG. 8. The various arrangements of the prostomial mucus-gland system found in the Nephthyidae. The first three species in the upper row show an increasing development of the prostomial epidermal mucus-glands; those in the lower row indicate stages in the centripetal migration of epidermal mucus-cells into the posterior lobes and into the anterior prostomial group.

dorsal, or more usually, to the ventral surface of the prostomium. In *N. caeca*, *N. ferruginea*, *N. parva*, *N. punctata*, and *N. incisa*, where the latter condition obtains, there are no anterior lateral organs and their place is taken by an anterior group of epidermal cells. This is almost the condition found in *N. longosetosa*, but a few of the anterior median mucus-cells open to lateral walls of the prostomium, forming a small anterior lateral organ in addition to a small anterior group of mucus-cells in the lateral walls. Posterior lobe cells invariably open to the exterior by way of the posterior lateral organs. *N. incisa* and *N. cornuta* do not have posterior lobes and consequently lack lateral organs, but the place of the latter is taken by epidermal mucus-cells. *N. picta* and *Aglaophamus* spp. have very few epidermal mucus-cells in the prostomium and the only recognizable group of them is in the median anterior area. These open directly to the exterior.

The anterior and posterior parts of the prostomial mucus-gland system vary independently and the degree of development of epidermal mucus-glands also differs markedly in different species.

The various forms the system may take are summarized in the following table and in fig. 8.

	Post. lobes	Post. lat. organs	Post. lat. mucus-cells	Ant. median prostomial group	Ant. lat. organs	Ant. lat. mucus- cells
<i>N. californiensis</i>	×	×	—	×	×	—
<i>N. caecoides</i>	×	×	—	×	×	—
<i>N. longosetosa</i>	×	×	—	×	×	×
<i>N. caeca</i>	×	×	—	×	—	×
<i>N. ferruginea</i>	×	×	—	×	—	×
<i>N. glabra</i>	×	×	—	×	—	×
<i>N. magellanica</i>	×	×	—	×	—	×
<i>N. parva</i>	×	×	—	×	—	×
<i>N. rickettsi</i>	×	×	—	×	—	×
<i>N. punctata</i>	×	×	—	×	×	—
<i>N. cornuta</i>	—	—	×	×	×	—
<i>N. cornuta franciscana</i>	—	—	×	×	—	×
<i>N. incisa</i>	—	—	×	×	—	×
<i>N. squamosa</i>	—	—	×	×	—	×
<i>N. hombergi</i>	—	—	—	×	—	—
<i>N. picta</i>	—	—	—	×	—	—
<i>A. dicirris</i>	—	—	—	×	—	—
<i>A. erectans</i>	—	—	—	×	—	—
<i>A. virginis</i>	—	—	—	×	×	—
<i>N. cirrosa</i>	*	*	—	×	×	—

* See separate discussion of this species.

THE POSTERIOR LOBES OF *NEPHTYS CIRROSA*

N. cirrosa represents a special case and must be described in detail. The supra-oesophageal ganglion lies in the posterior part of the prostomium and in the first segment. A pair of lobes extends from its posterior border into the anterior part of the fourth segment (fig. 9). The histological appearance of

these lobes differs markedly from that of any other species. The lobe itself is separated from the ganglion proper by a barrier of neuroglial fibres as in other species with posterior lobes. There is also a dense penetration of neuroglia into the posterior lobes, quite different from the occasional neuroglial fibres which penetrate into the posterior lobes of a species such as *N. californiensis*. Secretory cells are scattered among the neuroglia, together with the neuroglial cell-bodies and larger matrix cells (fig. 4, c). The cell-bodies of the secretory

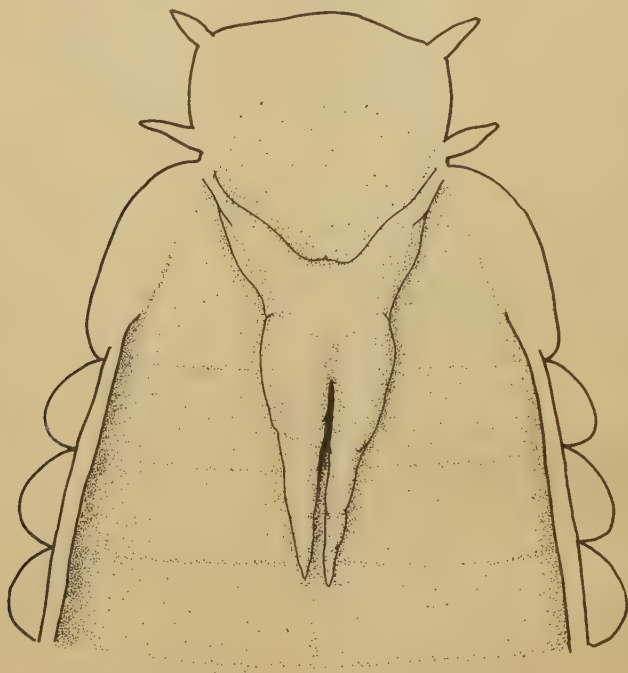


FIG. 9. Supra-oesophageal ganglion and posterior lobes of *N. cirrosa*. Dissection from the ventral side.

cells are about 15μ long by 8μ wide and are packed with strongly fuchsinophil granular inclusions, which are often so numerous and dense as to obscure the structure and form of the cell. These cells are most numerous in the posterior ends of the lobes; a few are to be found along the lateral edges of the lobes as far forward as the posterior end of the brain.

The processes from the posterior lobe secretory cells of *N. cirrosa* are much finer than those of *N. californiensis* and can only be traced by the course of the granules. They run along the sides of the posterior lobes and the supra-oesophageal ganglion to the anterior margin of the latter. Then they run along the outer edges of the circum-oesophageal connectives, which are in contact with the epidermal cells of the lateral prostomial walls. At the point where the circum-oesophageal connectives turn sharply in a ventral direction, the fuchsinophil granules can be seen running through the epidermis to the

cuticle. Morphologically, the disposition of the cells and processes that are positive to paraldehyde fuchsin is essentially the same in *N. cirrosa* as in *N. californiensis* (fig. 10). There are far fewer secretory cells in the posterior lobes of *N. cirrosa* and their processes are much narrower, so that in consequence there are no conspicuous lateral organs, but processes from posterior lobe cells run through the epidermis in the position where lateral organs would be

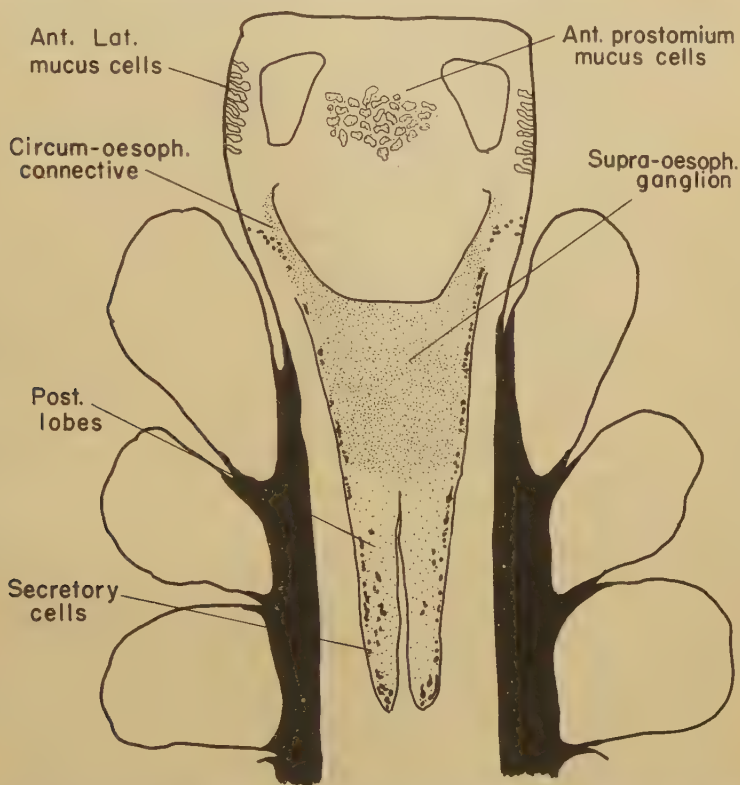


FIG. 10. Composite frontal section through the anterior end of *N. cirrosa* to show the epidermal mucus-glands of the prostomium and the path taken by granules from secretory cells in the posterior lobes. Based on camera lucida drawings.

expected to be found. Epidermal mucus-cells of the posterior lateral group adjoin the terminations of the fibres from the posterior lobe cells. The mucus-glands of the median anterior group open to the exterior in the lateral walls of the prostomium, forming an anterior lateral organ.

DISCUSSION

From these descriptions of the arrangement of the posterior lobes and their associated structures in the Nephtyidae, it appears that we have to deal with a system of epidermal mucus-cells which have become more or less incorporated with the supra-oesophageal ganglion. It is a simple matter to arrange the

species in a series showing progressive stages in a centripetal migration of epidermal mucus-cells. In a species such as *N. picta* or *Aglaophamus* spp. there are relatively few mucus-cells in the prostomium and those that there are are embedded in the epidermis and open directly to the exterior. At the opposite end of the series we may place *N. californiensis* or *N. caecoides*. In these worms there are no mucus-cells in the epidermis of the prostomium; instead they are concentrated in the posterior lobes and the anterior prostomial group and communicate with the exterior by long processes terminating in the lateral organs. Intermediate in the series are *N. cornuta*, in which the anterior prostomial mucus-cells open to the exterior in the lateral walls of the prostomium and there are no antero-lateral epidermal mucus-cells and no posterior lobes, and *N. caeca*, in which there are posterior lobes and posterior lateral organs, but cells of the median anterior prostomial group open directly to the exterior. It seems likely that the epidermal mucus-cells in the anterior lateral walls of the prostomium have migrated into the anterior median group, and those in the posterior part of the lateral walls have migrated into the posterior lobes. Both groups of mucus-cells open to the exterior in the lateral walls of the prostomium whether they are in their original peripheral position or if they have migrated centrally. To judge from the disposition of the mucus-cells in the species illustrated in fig. 8, the two processes have gone on independently.

The fact that 20 or so species can be arranged in such an order that a sequential elaboration and integration of the mucus-gland system can be demonstrated does not, of course, prove that the evolution of these structures has taken the same course. If it had, one might expect to find more intermediate cases in the postulated series. The hypothesis would receive strong support if a species were discovered in which there was a group of mucus-cells lateral to the supra-oesophageal ganglion in the position occupied by the lateral tract of processes from the posterior lobe cells in *N. californiensis*. However, this intermediate condition does not exist in any of the 21 species I have examined and the nearest approach to such a condition is that found in *N. californiensis*, in which a few mucus-cells are scattered along the lateral tracts of cell processes.

While the mucus-cells of the posterior part of the prostomium apparently occur either in the epidermis or in the posterior lobes, but not in intermediate positions, those of the anterior part of the prostomium are not so uncompromisingly divided into peripheral or central groups. In most species those in the middle of the prostomium open directly to the exterior either dorsally or ventrally or both, and the anterior lateral mucus-cells are not related to those of the central group. In *N. longosetosa* a few cells in the median group do not open to the exterior ventrally, as the rest of them do, but by way of long processes to the sides of the prostomium. This species has still a small group of anterior lateral mucus-cells in addition to the incipient lateral organ. In *N. californiensis* and *N. caecoides* the anterior lateral mucus-cells are completely replaced by the anterior part of the lateral organ and all the cell-bodies are

located in the central mass or along the course of the cell processes. Some of the mucus-cells in the median mass of both these species still open directly to the dorsal or ventral surfaces of the prostomium.

Although the posterior part of the prostomial mucus-gland system comes to have an intimate connexion with the supra-oesophageal ganglion, the anterior part has no connexion with any part of the nervous system. In spite of this it seems justifiable to treat both as parts of the same system, particularly in view of the fact that in what we have postulated to be the primitive condition, found in such a species as *N. incisa*, it is impossible to distinguish between the anterior and posterior groups of mucus-cells in the lateral walls of the prostomium. At first sight it may seem surprising that epidermal mucus-cells should have been incorporated in the brain to the extent that they are contiguous with the nervous tissue and are enclosed within the membranes which invest the ganglion. There is, however, a precedent for this in the evolution of the cerebral organs of nemerteans, which is strikingly similar to the postulated evolution of the posterior lobes of *Nephtys*.

The cerebral organs of nemerteans are partly ganglionic and partly glandular. Their structure had been known for some time, but no detailed and comparative account of them had been given until Scharrer (1941) made a study of the structure of those of *Lineus* and *Cerebratulus*, in which they are incorporated within the brain capsule. She proposed that they had evolved from epidermal structures. In *Carinella annulata* the ganglion cells and glandular cells of the cerebral organ are purely epidermal and are connected with the cerebral ganglion by a long nerve running through the muscle layers of the body-wall. In *Derpanopus albolineatus* the cerebral organ is internal to the muscle layers but connected to the exterior by a cerebral canal. In *Amphiporus marmoratus*, *Lineus coecineus*, and *Cerebratulus lacteus* the cerebral organ is associated closely with the cerebral ganglion and shows a progressively greater degree of incorporation within it in the three species. In the first it is in contact with the ganglion, but still appears as a separate structure. In the other two it is completely within the connective tissue sheath of the cerebral ganglion. In *Cerebratulus* there is an uninterrupted transition from ganglion cells to glandular cells in the posterior and antero-lateral parts of the brain.

Scharrer was able to cite embryological evidence of the epidermal origin of the cerebral organs of *Lineus* in support of her thesis. The hypothesis that the posterior lobes of *Nephtys* are derived from epidermal mucus-cells would be greatly strengthened by embryological evidence of the migration of cells from the epidermis into the lobes and the anterior median group of mucus-cells. Unfortunately no detailed study of the embryology of any nephtyid has ever been carried out. Until it has been, an analysis of the evolution of these structures must be based on a consideration of comparative anatomy alone. The evidence for the thesis, then, amounts to the following:

1. Cells in the prostomial epidermis, in the median anterior group, in the posterior lobes, and in the parapodial mucus-glands are identical in appearance.

2. All respond in the same way to the histochemical tests discussed above and all secrete an acid mucopolysaccharide.
3. All these structures are epidermal and are bounded internally by the basement membrane of the epidermis or by extensions of it.
4. A sequence can be discerned in the species examined which is consistent with the view that a centripetal migration of these cells has taken place.
5. A strikingly similar example of the phylogenetic and ontogenetic centripetal migration of epidermal glandular cells with a subsequent incorporation in the cerebral ganglion has been reported in the nemerteans.

N. cirrosa represents a separate problem and possibly illustrates a further stage in the evolution of the posterior lobes and a closer association of the cells in them with the supra-oesophageal ganglion. However, the histological appearance of the lobes of this species differs so markedly from that of the posterior lobes of any other, that any discussion of them must be tentative for the present. Perhaps after the investigation of the histology of the lobes has been extended to a greater number of species of *Nephtys* and the nature of the secretion produced by the cells in the posterior lobes of *N. cirrosa* is known, it will be possible to be more positive about them. Only one of the three types of cell in the posterior lobes of *N. cirrosa* secretes fuchsinophil granules and they are much smaller than the secretory cells in the posterior lobes of *N. californiensis* and have a quite different appearance. In fact, they look much more like the neurosecretory cells of the supra-oesophageal ganglion. The question arises, are the posterior lobes of *N. cirrosa* homologous with those of *N. californiensis*? There are three possible origins of the cells in the lobes of the former species:

1. They cannot be homologized with any feature of the brain of any other nephtyid and the resemblances are the result of convergence. In other words, the posterior lobes of *N. cirrosa* have developed *de novo*.
2. They represent ganglion cells of the posterior part of the supra-oesophageal ganglion which have migrated caudally into the neuroglial area at its posterior end. The whole of the posterior part of the brain has therefore hypertrophied.
3. They represent modified posterior lobe cells, and the posterior lobes of *N. cirrosa* can be homologized with those of *N. californiensis* and other nephtyids.

As to the first alternative, the appearance of a completely new nervous structure, with no hint of its existence in any other species, is not likely. While this possibility cannot be excluded, particularly since only about a third of the species in the family have been examined, to admit it on so slight evidence would be to deny the principles of comparative morphology.

The second alternative, that the posterior region of the supra-oesophageal ganglion has hypertrophied in this species, is at first sight the most attractive. However, in spite of the great variation in the fine structure of the supra-

oesophageal ganglion of *Nephtys*, the arrangement of groups of neurones in the posterior part of the brain is one of the few constant features. There are two large groups of nerve-cells in the posterior part of the brain of all species of *Nephtys*, which are probably homologous with a similar group in the posterior part of the brain of Nereids also. In some species of *Nephtys* they may extend part of the way into the anterior part of the posterior lobes, but they are always separated from the secretory cells of the lobes by a barrier of neuroglial fibres. The neurones of these two groups are neurosecretory in all species of *Nephtys* examined (Clark, unpublished data) and also in *Nereis* (Scharrer, 1936). These are represented in the posterior part of the ganglion, and not in the posterior lobes, of *N. cirrosa*. In addition there are four large neurosecretory cells of a different type, lateral to the two posterior groups of cells (Clark, unpublished data). These, too, are present in the ganglion of *N. cirrosa*. Finally, the eyes of *Nephtys*, which are located in this region of the ganglion and bear a constant relation to the neurosecretory cells of both types, occur in their usual position in *N. cirrosa* (Clark, 1956b). Thus all the recognizably constant features of the posterior part of the supra-oesophageal ganglion of other species of *Nephtys* occur also in *N. cirrosa* in their typical positions and not in the posterior lobes. For this reason it is difficult to maintain that the posterior lobes of this species represent a hypertrophy of the posterior part of the supra-oesophageal ganglion.

We are thus forced to consider the third alternative, that the posterior lobes *N. cirrosa* and *N. californiensis* are homologous. In favour of this view, the fibres from the secretory cells of the posterior lobes of the former species run in the same place and open to the exterior in the same place as they do in *N. californiensis*. In addition, both produce fuchsinophil granules of secreted material. On the other hand, the secretory cells in the posterior lobes of *N. cirrosa* form but a small minority of the cells and they look nothing like those of the posterior lobes of other species of *Nephtys*. They look remarkably like the majority of the neurosecretory cells of the supra-oesophageal ganglion. Whether or not there are neurosecretory cells in the posterior lobes of *N. cirrosa* can only be determined after detailed study of the histology of these cells and of the histochemistry of the secretion produced by these and the neurosecretory cells of the supra-oesophageal ganglion. An investigation of this sort is now in progress.

If this analysis is correct, the posterior lobes of *N. cirrosa* demonstrate a remarkable incorporation of epidermal glandular cells within the central nervous system. Even in the nemerteans, the glandular tissue of the cerebral organs is recognizably of the same histological appearance whether it is epidermal, as in *Carinella*, or completely incorporated within the cerebral ganglion, as in *Cerebratulus* (Scharrer, 1941). This is also true of most nephtyids, but in *N. cirrosa* the secretory cells of the posterior lobes appear to have become completely integrated with the nervous system. Indeed, were it not for a knowledge of the structure of the posterior lobes and their probable evolution of other nephtyids, one would certainly not attempt to distinguish

between the posterior lobes of *N. cirrosa* and the rest of the supra-oesophageal ganglion.

The function of the mucus-gland system of the prostomium is unknown. The cells of the posterior lobes of *N. caecoides*, in which the lobes reach their greatest development, produce copious quantities of mucus which appears to be readily discharged to the exterior. Whatever its function, it must be of considerable biological significance. The fact that the prostomial mucus-glands are so poorly developed in some species, e.g. *N. picta*, suggests that there has been a great elaboration of some activity of *Nephtys* in the course of the evolution of this worm. Unfortunately, practically nothing is known of the ecology of the Nephtyidae and it is impossible to speculate on the function of this glandular system. One or two possible functions can be excluded, however. One of the commonest functions of epidermal mucus-glands in polychaetes is the secretion of a tube in which the worm lives. In most species of *Nereis* this is done by the parapodial mucus-glands, but in *Nephtys* the epidermal mucus-glands in the segmental part of the body are comparatively poorly developed and the worm does not secrete a tube, nor does it consolidate its burrow in the sand with mucus. Several polychaetes lay their eggs in mucous capsules and this could conceivably be a function of the prostomial mucus-glands of *Nephtys*. But Augener (1912) has described epitokous forms of a number of species of *Nephtys*, and there is every indication that the worms swarm in the water for spawning and have pelagic eggs and larvae. Finally, mucus is sometimes secreted to form a food-trapping net, as in the chaetopterids. This seems unlikely in *Nephtys* because it has no permanent burrow and is probably a carnivore. This possibility must be rejected with caution, though, since *Nereis diversicolor*, regarded as a typical carnivorous polychaete, has been observed to secrete a mucous net for just this purpose (Harley, 1950).

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